CANADIAN THESES ON MICROFICHE

I.S.B.N.

THESES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

NL-339 (r. 82/08)

Canada

National Library of Canada
Canadian Theses Division
Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Division des thèses canadiennes

0-315-06059-X

53979

PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

- Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur

Brian Thomas McDonough

Date of Birth — Date de naissance

13/04/56

Country of Birth — Lieu de naissance

Canada

Permanent Address — Résidence fixe

1257 Normandie St. Hubert Quebec J4T 1H9

Title of Thesis — Titre de la thèse

The effects of Fatty Acid Structure on Acyl Chain Organization in Biological Membranes: an ^{19}F-NMR Study.

University — Université

University of Alberta.

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Master of Science

Year this degree conferred — Année d'obtention de ce grade

1981

Name of Supervisor — Nom du directeur de thèse

Ronald McElhaney

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

Date July 10th, 1981

Signature Brian McDonough.

NL-91 (4/77)

THE UNIVERSITY OF ALBERTA

The Effects of Fatty Acid Structure on Acyl Chain Organization in Biological Membranes: An ^{19}F-NMR Study

by

© Brian McDonough

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF Master of Science

Biochemistry

EDMONTON, ALBERTA
Fall 1981

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR Brian McDonough

TITLE OF THESIS The Effects of Fatty Acid Structure on Acyl Chain Organization in Biological Membranes: An ^{19}F-NMR Study

DEGREE FOR WHICH THESIS WAS PRESENTED Master of Science

YEAR THIS DEGREE GRANTED July 1981

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) Brian McDonough

PERMANENT ADDRESS:

1257 Normandie street
St. Hubert, Quebec
J4T 1H9

DATED July 3rd 1981

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled The Effects of Fatty Acid Structure on Acyl Chain Organization in Biological Membranes: An ^{19}F-NMR Study submitted by Brian McDonough in partial fulfilment of the requirements for the degree of Master of Science.

Ronald N. McElhaney

Supervisor

Brian D. Sykes

Mark Lazowsky

Date July 28, 1981

To my Parents and Alexandra

ABSTRACT

The objective of this work was threefold. Firstly, the synthesis of specific monofluoropalmitic acid analogs. Secondly, an evaluation of these monofluorinated fatty acids as ^{19}F-NMR probes of lipid bilayers. Thirdly, a study of the effect of fatty acid structure and temperature on the orientational order of the hydrocarbon chains through the lipid bilayer of a biological membrane, specifically, the plasma membrane of *Acholeplasma laidlawii* B, using these monofluoropalmitic acid analogs as ^{19}F-NMR probes.

The similarity in size of the fluorine and hydrogen atoms, the low reactivity of the carbon-fluorine bond in an alkyl chain, and the high sensitivity of ^{19}F-NMR spectroscopy suggest that the specifically monofluorinated fatty acids may be useful NMR probes of membrane structure. To evaluate the possible perturbation of the lipid bilayer by the fluorine atom, the thermotropic phase behavior of a series of di-monofluoropalmitoyl phosphatidylcholines were studied, and the results indicate that their properties closely resembled those of the non-fluorinated analogue, dipalmitoyl phosphatidylcholine. In addition, the incorporation of up to 30 mole % of various monofluoropalmitic acids into the membrane lipids of *Acholeplasma laidlawii* B did not adversely affect the growth of this organism. At higher levels of incorporation, these fluorinated fatty acids progressively decreased growth yields, with this effect being greatest for the isomers with the fluorine atom substituted nearest the carbonyl group of the fatty acid. Moreover, the levels of incorporation of these fluorinated fatty acids were similar to that of palmitic acid. Finally, the determined order parameters of the monofluoropalmitic acids incorporated into palmitic acid-enriched

membranes containing various levels of the probes were constant, suggesting the monofluoropalmitic acids closely mimicked the physical properties of palmitic acid in the membrane of this organism. Taken together, these results suggest that these monofluorinated fatty acids can be utilized as sensitive and relatively non-perturbing probes of fatty acyl chain organization in model and biological membranes.

The second aspect of this work was to study the effect of fatty acid structure and temperature on acyl chain order in a lipid bilayer. To this end I have collected ^{19}F-NMR spectra of Acholeplasma laidlawii B membranes containing predominantly one fatty acid plus a small amount of one of a number of the monofluoropalmitic acid probes. Spectra were collected above and (where possible) below the phase transition of the membrane lipids as well as at the optimum growth temperature (37°C). From these spectra an orientational order parameter ,Sz, was determined for a number of positions along the chain. The results indicate that the order is markedly dependent on both the acyl chain structure and the temperature. At the same temperature the presence of a methyl group near the methyl terminus of the chain decreases the average order, but increases the length of the plateau region relative to the straight-chain saturated fatty acids. In contrast, the presence of a trans-double bond decreases both the average order and the length of the plateau region. Similar results were observed below the phase transition, while at a fixed temperature above the phase transition of the membrane lipids relative to the midpoint of the phase transition, the average order in the presence of a methyl group and a trans-double bond increases relative to when these groups are not present. These results suggest that by controlling the structure of its membrane fatty acids an organism may control both the absolute order and the order profile of the hydrocarbon core of its lipid bilayer.

ACKNOWLEDGEMENTS

It is with pleasure that I take this opportunity to thank the many people who have made the time spent doing this work both an interesting and stimulating experience. I would like to express thanks to my supervisor, Dr. Ronald McElhaney, whose knowledge and patience encouraged this work, and helped alleviate the stress associated with graduate studies, as well as for the squash lesson which proved there is life after thirty. To Dr. Brian Sykes who was intimately involved in all aspects of the NMR studies and contributed greatly to my understanding in this area. I give special thanks to my lab cohorts, Dr. John Silvius who introduced me to the wonders of organic chemistry, Dr. Ruthven Lewis whose many helpful discussions and timely suggestions, scientific or otherwise, enhanced this work, and Ms. Nanette Mak whose calming influence and technical efficiency managed to keep the scientific ball rolling and introduced me to the wonders and horrors of Chinese food. I also express sincere thanks to Mrs. Dawn Oare who transformed my written word with its numerous spelling errors into coherent type-written order, and Perry D'Obrenan who drafted the figures in this thesis. I also thank Dr. E. E. Burnell at the University of British Columbia and the Department of Chemistry at this university for the use of their NMR facilities for the field-dependent studies. I also thank the many people, too numerous to mention, who have helped me along the way and made this work and the time spent here an enjoyable experience. Finally, I thank the Department of Biochemistry and the Alberta Heritage Trust Fund for Medical Research for financial support during this work.

Table of Contents

Chapter Page

I. INTRODUCTION 1

A. Infrared and Raman Spectroscopy 5

B. Fluorescence Spectroscopy 6

C. Electron Spin Resonance Spectroscopy 10

D. Nuclear Magnetic Resonance Spectroscopy 14

II. MATERIALS AND METHODS 19

A. Materials 19

B. Methods 20

C. Synthesis of Fluorinated Lipids 23

D. Theory of Lineshape Analysis 26

III. EVALUATION OF THE USEFULNESS OF MONOFLUORINATED PALMITIC ACIDS AS NMR PROBES OF MEMBRANE STRUCTURE 30

A. Introduction 30

B. Results and Discussions 31

Growth Studies with Acholeplasma laidlawii B 38

Nuclear Magnetic Resonance Experiments 41

C. Conclusions 47

IV. THE EFFECT OF FATTY ACID STRUCTURE AND TEMPERATURE ON THE ORDER OF THE ACYL CHAINS IN ACHOLEPLASMA LAIDLAWII B MEMBRANES AS SEEN WITH ^{19}F-NMR 50

A. Introduction 50

B. Results and Discussion 51

C. Conclusions 84

V. BIBLIOGRAPHY 87

VI. APPENDIX 1 92

VII. APPENDIX 2 98

LIST OF TABLES

Page

1. - The thermotropic phase transition properties of the pure di-monofluoropalmitoyl Phosphatidylcholines. 32

2. - The thermotropic phase transition properties of mixtures of the di-monofluoropalmitoyl Phosphatidylcholines and dipalmitoyl Phosphatidylcholine 37

3. - Fatty acid composition of A. laidlawii B membranes from cells cultured in media supplemented with 85% palmitic acid and 15% of one of the monofluorinated palmitic acid analogs 39

4. - Fatty acid composition of membranes enriched with iso-palmitate containing 10% of each of the monofluoropalmitic acid probes from cells

grown in the presence of avidin 53

5. - Fatty acid composition of membranes enriched with anteiso-palmitate containing 10% of each of the monofluoropalmitic acid probes from cells grown in the presence of avidin 54

6. - Fatty acid composition of membranes enriched with Palmitelaidate containing 10% of each of the monofluoropalmitic acid probes from cells grown in the presence of avidin 55

7. - Fatty acid composition of membranes enriched with Elaidate containing 10% of each of the monofluoropalmitic acid probes from cells grown in the presence of avidin 56

8. - Fatty acid composition of membranes enriched with Pentadecanoic acid containing 10% of each of the monofluoropalmitic acid probes from cells grown in the presence of avidin 57

Page

9. - The NMR parameter values that give the best fit of the theoretical lineshapes to the observed spectra of identical samples collected at different magnetic field strengths 64

LIST OF FIGURES

Page

1. - The structure of some of the more common spectroscopic probes of lipid bilayers 8

2. - The order parameter profiles of lipid bilayers observed with the different spectroscopic techniques 11

3. - The synthetic pathway used to synthesize the monofluoropalmitic acid analogs from the corresponding keto-acid methyl ester 25

4. - The phase transition curves observed for the di-monofluoropalmitoyl Phosphatidylcholines and mixtures of these with dipalmitoyl Phosphatidylcholine 34

5. - The effect of increasing concentration of the monofluoropalmitic acid analogs in the membrane of cells supplemented with palmitic acid as measured by total cell yield 40

6. - The effect of increasing concentration of the monofluoropalmitic acid analogs in the membrane of cells supplemented with various fatty acids capable of supporting good growth in the presence of avidin as measured by total cell yield 42

7. - The order parameter profiles obtained from _A. laidlawii_ B membranes from cells enriched with specifically deuterated or fluorinated palmitic acid analogs 44

8. - The phase transition curves for _A. laidlawii_ B membranes enriched with palmitic acid and with palmitic and 14-fluoropalmitic acid in a one-to-one ratio 45

9. - The order parameters determined from palmitic acid-enriched membranes containing various concentrations of either 14- or 5-fluoropalmitic acid at temperatures above, below, and in the region of the phase transition of the membrane lipids 48

Page

10. - The effect of changes in the parameter Δo on the theoretical lineshapes 60

11. - The NMR spectra of membranes enriched with palmitic acid containing 5-fluoropalmitic acid collected at different field strengths 62

12. - The NMR spectra of membranes enriched with palmitelaidic acid containing 14-fluoropalmitic acid collected at different field strengths 63

13. - The effect of changes in the order parameter ,Sz, on the theoretical lineshapes 66

14. - The order profiles of straight-chain saturated and methyl branched fatty acid-enriched membranes at the growth temperature (37°) 67

15. - The order profiles of straight-chain saturated and methyl branched fatty acid-enriched membranes at 15° above the Tm of the membrane lipids 69

16. - The order profiles of straight-chain saturated and trans-unsaturated fatty acid-enriched membranes at the growth temperature (37°) 72

17. - The order profiles of straight-chain saturated and trans-unsaturated fatty acid-enriched membranes at 15° above the Tm of the membrane lipids 73

18. - The order profiles of straight-chain saturated and methyl branched fatty acid-enriched membranes below the phase transition of the membrane lipids or 5°C 74

19. - The order profiles of straight-chain saturated and trans-unsaturated fatty acid-enriched membranes below the phase

Page

transition of the membrane lipids or 5°C 75

20. - The order profiles of palmitic acid-enriched membranes at various temperatures 78

21. - The order profiles of pentadecanoic acid-enriched membranes at various temperatures 79

22. - The order profiles of iso-palmitic acid-enriched membranes at various temperatures 80

23. - The order profiles of anteiso-palmitic acid-enriched membranes at various temperatures 81

24. - The order profiles of palmitelaidic acid-enriched membranes at various temperatures 82

25. - The order profiles of elaidic acid-enriched membranes at various temperatures 83

ABBREVIATIONS AND SYMBOLS

AN	1-anilino-8-naphthalene
ANS	1-anilino-8-naphthalene sulfonate
°C	degrees centigrade
CUS	cooperative unit size
DSC	differential scanning calorimetry
DPH	diphenylhexatriene
ESR	electron spin resonance
FID	free induction decay
GLC	gas-liquid chromatography
g	grams
ΔHcal	calorimetric enthalpy in kilocalories
$\Delta H_{v}H$	van't Hoff enthalpy in kilocalories
Hz	hertz
hr	hour
I.R.	infrared spectroscopy
I.U.	international units
°K	degrees kelvin
l	liter
M	mole
M-,m-,u-	mega-, milli-, micro (10^{6}, 10^{-3}, 10^{-6})
min	minute
NMR	nuclear magnetic resonance
ONS	n-octadecylnaphthyl-2-amino-6-sulfonic acid
PC	phosphatidylcholine
sec	second
Sz	order parameter

T	temperature
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TLC	thin-layer chromatography
Tm	main transition midpoint temperature
Tp	pre-transition midpoint temperature
VPBO	2,2'-(Vinylenedi-*p*-phenylene)bisbenzoxazole

I. INTRODUCTION

Biological membranes consist primarily of lipids and proteins. Only in the past decade, after Singer and Nicolson (1972) proposed the fluid mosaic model for the molecular organization of biological membranes, have the structural and functional aspects of the individual components been understood (Singer, 1971). A typical cell membrane is composed of amphipathic lipid molecules organized into a lipid bilayer, with the hydrophilic headgroups exposed to the aqueous environment and the hydrophobic acyl chains interacting to form a hydrophobic bilayer core. The proteins are "dissolved" in the lipid matrix and may be classified as either peripheral or integral membrane proteins. These two classes may be distinguished by the way they interact with the lipid bilayer. Peripheral proteins interact primarily through electrostatic interactions with the lipid headgroups. In contrast, integral proteins also exhibit large interactions with the hydrophobic core of the bilayer. Integral proteins may be further classified as either trans-membrane proteins which span the lipid bilayer, or proteins that are only partially embedded into one side of the bilayer. As a consequence of these differences between periphal and integral membrane proteins, they are easily distinguished experimently. Peripheral proteins may be extracted from the membrane by gentle sonication or by high salt concentrations, whereas the extraction of integral proteins requires more vigorous treatments such as the use of detergents. The lipid bilayer may be thought of as the permeability barrier for the cell, while the membrane proteins function as active transport systems, enzymes, hormone receptors and other things associated with membrane activities.

One of the many interesting questions raised by this model concerns the structural and dynamical properties of the lipid bilayer. An enormous heterogeneity of lipids have been found to exist in membranes, indeed even within the same membrane (Guidotti, 1972). Questions as to how this heterogeneity is involved with the regulation of membrane protein activity, lipid interactions, and membrane properties encompass a major portion of present day membrane research. I will concentrate my review primarily on the membrane lipids, specifically on the organization and dynamics of the lipid molecules in a lipid bilayer.

In the past fifteen years a large amount of research has been done in this area with a wealth of information coming from spectroscopic techniques (Anderson, 1978). The major question addressed in these studies is how the rates of motion of the lipid molecules are related to the structure of these molecules. In order to address this question the ideas of fluidity and order of the acyl chains in a lipid bilayer must be understood.

A great deal of confusion in membrane research has resulted from the ambiguity of the term "fluidity" (Schreier _et_ _al._, 1978). For an isotropic solution, the fluidity is defined as the reciprocal of the viscosity. Within the confines of this strict definition, the concept of fluidity cannot be strictly applied to an anisotropic system such as a lipid bilayer, which exhibits restricted motion and partial ordering of the lipid molecules. In an axially symmetric system such as a lipid bilayer (Seiter and Chan, 1973; Lichtenberg _et_ _al._, 1975), fluidity is described as a measure of the different rates of motion of the lipid molecules, which cannot be described by one correlation time as was the case for an isotropic system, but is composed of a number of different correlation times defining the motion of the headgroups, rotation of the acyl chains around its long axis, chain tilting within the bilayer and trans-gauche isomerization about carbon-carbon bonds. Thus measurements of these different correlation times gives information about the dynamics of the lipids within the bilayer.

A second aspect to be considered is the organization of the lipids in the bilayer. A structural order parameter may be derived that is a measure of the average position of a particular region of an acyl chain with respect to the axis of symmetry, in this case the bilayer normal. For a completely ordered bilayer a value of one is assigned to the order parameter,Sz, which means that all the chains are in the all-trans conformation and are arranged parallel to the bilayer normal. A value of zero for Sz implies a completely "random" orientation of the chain with respect to the bilayer normal. This "random" conformation may be the result of the isotropic averaging of the molecules (fast motion) or due to the "immobilization" on the NMR time scale of the acyl chains in a highly disordered state (slow motion) which may occur in the case of protein-lipid interactions in the bilayer (Jost et al., 1978; Kang et al., 1979). Thus values of the order parameter between these two limits are a measure of the average deviation of the chain segment from the bilayer normal. This definition of the order of a system does not require any knowledge of the rates of motion of the molecules, but only that these motions are not isotropically averaged within the time frame of the experiment. Although the order parameter is a structural parameter, it is not completely independent of time. If a motion is fast enough to isotropically average particular structural conformations, then the measured order will not include any contributions from this conformation. However, if the experiment is repeated where the time scale is shorter and this conformation is not isotropically averaged, then a different value of the order may be observed. Such a situation may arise when one measures the order with nuclear magnetic resonance (NMR) and electron spin resonance (ESR), and must be taken into account when comparing order parameter values obtained using the two techniques.

A final point to consider is that for a system in which Sz is greater than zero, the components must be undergoing motional restriction or partial ordering. The absolute value observed for the order parameter does not contain information about the absolute rates of motion or fluidity; however, for a particular system, such as a lipid bilayer, an increase or decrease in the order parameter (as a result of a decrease or increase in the temperature or the addition of cholesterol) tells us that the degree of motional restriction experienced by the lipid molecules has changed (Peterson and Chan, 1977), which in turn will be reflected in some or all of the rates of motions of the lipids. Thus the relative changes in the values of Sz observed for a particular system using the same experimental technique will correlate with the relative changes in the fluidity, where an increase in the observed order due for example to a decrease in the temperature, implies that there has been a relative decrease in the fluidity.

A number of spectroscopic techniques have been used to study the structure and dynamics of acyl chains in bilayer systems. In the following brief review I present the information that has been obtained from these techniques as well as the advantages and disadvantages inherent in each.

A. Infrared and Raman Spectroscopy

Infrared and Raman spectroscopy sample the vibrational transitions of molecular bonds, which occur on a time scale in the range of 10^{-11} seconds. Since these times are much faster than any of the motions experienced by the lipid molecules (translation, rotation, or isomerization), this technique gives us a static picture of the bilayer, which contains no information about the rate of motion of the molecules. There is also a large amount of spectral overlap of a number of the relevant vibrational transitions (Gaber *et al.*, 1978), which complicates the assignments of the spectral peaks. These problems may be partially overcome by the use of deuterated fatty acids, where the carbon-deuteron bond vibration can be differentiated from the carbon-hydrogen bond (Mendelson *et al.*, 1976); however, up to this point use of deuterated fatty acids have been limited primarily to simple model systems (Lavialle and Levin, 1980; Levin and Bush, 1981; Cameron *et al.*, 1980). The biggest disadvantage in the use of infrared spectroscopy is the large absorbance due to water, which when present obscures the transitions of interest. Water, however, does not affect the raman scattering experiment (Lippert and Peticolas, 1971). The advantages of this type of spectroscopy are that it is non-perturbing in that no probe needs to be added, and the vibrational transition frequencies are dependent on the conformation of the fatty acid chains (Gaber and Peticolas, 1977; Cameron *et al.*, 1980). Thus it is sensitive to the number of *gauche* conformers present in the acyl chain, which in turn can be related to the degree of order in the bilayer (Trauble, 1971; Gaber and Peticolas, 1977). These studies have shown that at temperatures well below the phase transition, where all the lipid is in the gel state, there still exists some disorder in the acyl chain packing, indicated by the presence of _gauche_ and "kink" conformers (Trauble, 1971). As the temperature is increased, the average number of these conformers gradually increases until the onset of the gel-to-liquid crystalline phase transition. At this point, there is a large decrease in the order of the chains and a large increase in the average number of _gauche_ conformers (Mendelson and Maisano, 1978). These observations agree with earlier x-ray diffraction and electron microscopic results which show an increase in the area occupied by the individual chains (Engelman, 1971), accompanied by a decrease in the bilayer thickness (Terry _et al._, 1967) as one goes from the gel to the liquid-crystalline state. These results are interpreted as a decrease in the average chain length due to the increase in the number of _gauche_ conformers present in the acyl chain in the liquid-crystalline state.

B. _Fluorescence Spectroscopy_

The major advantages of fluorescence and ESR spectroscopy are the high sensitivity of these probes and the relative ease with which the data obtained may be analyzed. In a fluorescence polarization experiment a probe-to-host lipid ratio in the range of 1 to 500 is sufficient to obtain good experimental results. When fluorescent probes are intercalated into lipid bilayers, they exhibit anisotropic motion. Thus when one measures the decay of the polarization of these probes, it is possible to extract the orientational and motional parameters of these probes, which in turn may be correlated to the orientation and dynamics of the probe environment (Shinitzky and Barenholz, 1978), within the time scale of 10^{-8} to 10^{-9} seconds.

These probes may be divided into three types. Probes such as 1-anilino-8-napthalene (AN) or diphenylhexatriene (DPH) are lipid soluble and freely diffusable within the bilayer, but do not have structures closely resembling those of natural lipids (see fig. 1). A second type is represented by n-(9-anthroyloxy)-fatty acids, in which the fluorescent group is covalently linked to a fatty acid, which may either be incorporated into a phospholipid or intercalated into the bilayer as a free fatty acid. Thirdly, probes such as the parinaric acids, which are naturally occurring, highly conjugated polyunsaturated fatty acids.

A major problem associated with the first type of probe is that their position in the bilayer is not known (Lentz et al., 1976). Because these probes are freely diffusable throughout the bilayer, differences detected in their fluorescence may reflect changes in the bilayer or may be the result of changes in the distribution of the probe within the bilayer. Recent results have indicatd that these probes may preferentially partition at lipid phase boundaries or around membrane proteins (Thulborn and Sawyer, 1978). This disadvantage may be overcome by the use of the other types of probes described above. In these cases the fluorescent group's position relative to the surface of the bilayer is fixed and only lateral diffusion of the probes occur (Thulborn and Sawyer; 1978, Lesshauer et al., 1972). Intuitively one might predict that the extent of perturbation introduced into a bilayer by a probe will be closely related to its size. This predicts that, with the exception of parinaric acid, fluorescent probes will be more perturbing than ESR probes (see fig. 1). Cadenhead and co-workers (1975), however, have shown that the amount of perturbation is not only a function of the

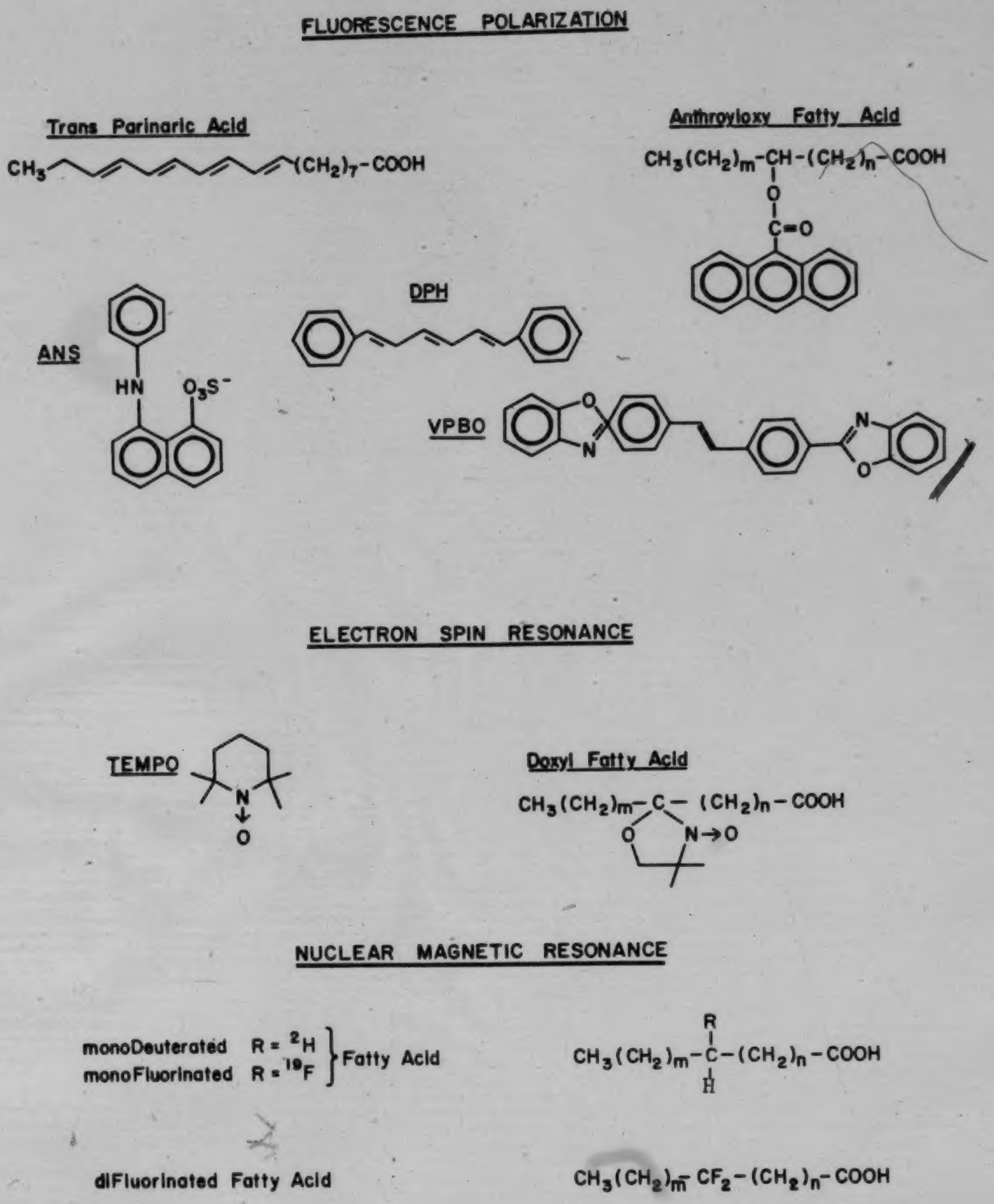

FLUORESCENCE POLARIZATION
Trans Parinaric Acid
CH3 (CH2)7-COOH
Anthroyloxy Fatty Acid
CH3(CH2)m-CH-(CH2)n-COOH
O
C=O
ANS
HN
O3S-
DPH
VPBO
O
N
N
O
ELECTRON SPIN RESONANCE
TEMPO
N
O
Doxyl Fatty Acid
CH3(CH2)m-C- (CH2)n-COOH
O
N→O
NUCLEAR MAGNETIC RESONANCE
monoDeuterated R = 2H
monoFluorinated R = 19F
Fatty Acid
R
CH3(CH2)m-C-(CH2)n-COOH
H
diFluorinated Fatty Acid
CH3(CH2)m-CF2-(CH2)n-COOH

Fig. 1. The structure of some of the commonly used spectroscopic probes of lipid bilayers.

size but also of the relative polarity of the probe. They did studies using a Wihelmy plate film balance, which measures changes in surface pressure and surface potential as a function of the surface area of the molecular film. They showed that 12-(9-anthroyloxy) stearic acid had the same surface potential as that of stearic acid, indicating that the anthroyloxy group was not exposed to the surface but remains buried in the hydrophobic region of the film. Mixed films of this probe and dipalmitoyl phosphatidylcholilne (PC) in a ratio of 1:50 showed significant negative deviations in the surface pressure when compared to pure dipalmitoyl PC films. This suggests the anthroyloxy group actually had a condensing effect on the PC film, leading to the conclusion that these probes have a marked effect on their microenvironment. Extensive studies on the behavior of fluorescent probes in lipid bilayers have been done (Bladely et al., 1973; Shinitzky et al.,1971; Shinitzky and Barenholz, 1978). The results observed in fluorescence polarization experiments were found to depend on the particular probe used (Bladely et al., 1973). The introduction of cholesterol into the lipid bilayer due to the addition of cholesterol (Butleret al., 1970) was found to have different effects on various probes. Cholesterol had hardly any effect on the apparent mobility of 12-(9-anthroyloxy) stearic acid, whereas it drastically reduced the mobility of the probe 2, 2'-Vinylenedi-p-phenylenebisbenzoxazole (VPBO) (see fig. 1), both probes of the hydrophobic region of the bilayers. In addition, for the two surface probes, n-octadecylnaphthyl-2-amino-6-sulfonic acid (ONS) and AN, cholesterol was shown to have opposite effects on their mobility. Cholesterol increased the mobility of ONS while it decreased the mobility of AN when it was present. Proton magnetic resonance studies have also been done in the presence of a number of different fluorescent probes to evaluate their effects on acyl chain properties (Podo and Blasie, 1977). Incorporation of 12-(9-anthroyloxy) stearic acid into dipalmitoyl PC liposomes was found to selectively increase the chemical shift of some of the methylene protons as well as decrease the T_1 values of these protons. Selective line broadening was also observed indicating that the probe reduces the motion of the methylene and methyl terminal portions of the acyl chains. These results agree with the molecular film studies previously discussed, which suggest this probe has a condensing effect on the neighbouring acyl chains when present in the bilayer. The 9-anthroyloxy derivatives of stearic and palmitic acid, where the anthroyloxy group has been covalently linked to various positions of the fatty acid chain, have been used to evaluate the mobility of the different segments of the chain in a bilayer (Tilley *et al.*, 1979; Thulborn and Sawyer, 1978). These studies reveal that the mobility of the probe increases linearly as its distance from the headgroup increases in PC liposomes (see fig. 2).

C. Electron Spin Resonance Spectroscopy

With the discovery of stable nitroxide groups, the use of ESR as a probe of membrane structure became a powerful technique (Hubbell and McConnell, 1971). The more commonly used probes are listed in fig. 1, and are somewhat analogous to the previously mentioned fluorescent probes. TEMPO is a freely diffusable probe similar to AN and DPH, while the fatty acids with the covalently linked nitroxide group resemble the n-(9-anthroyloxy) fatty acid probes. When these probes are intercalated into lipid bilayers they also exhibit anisotropic motion, and from the

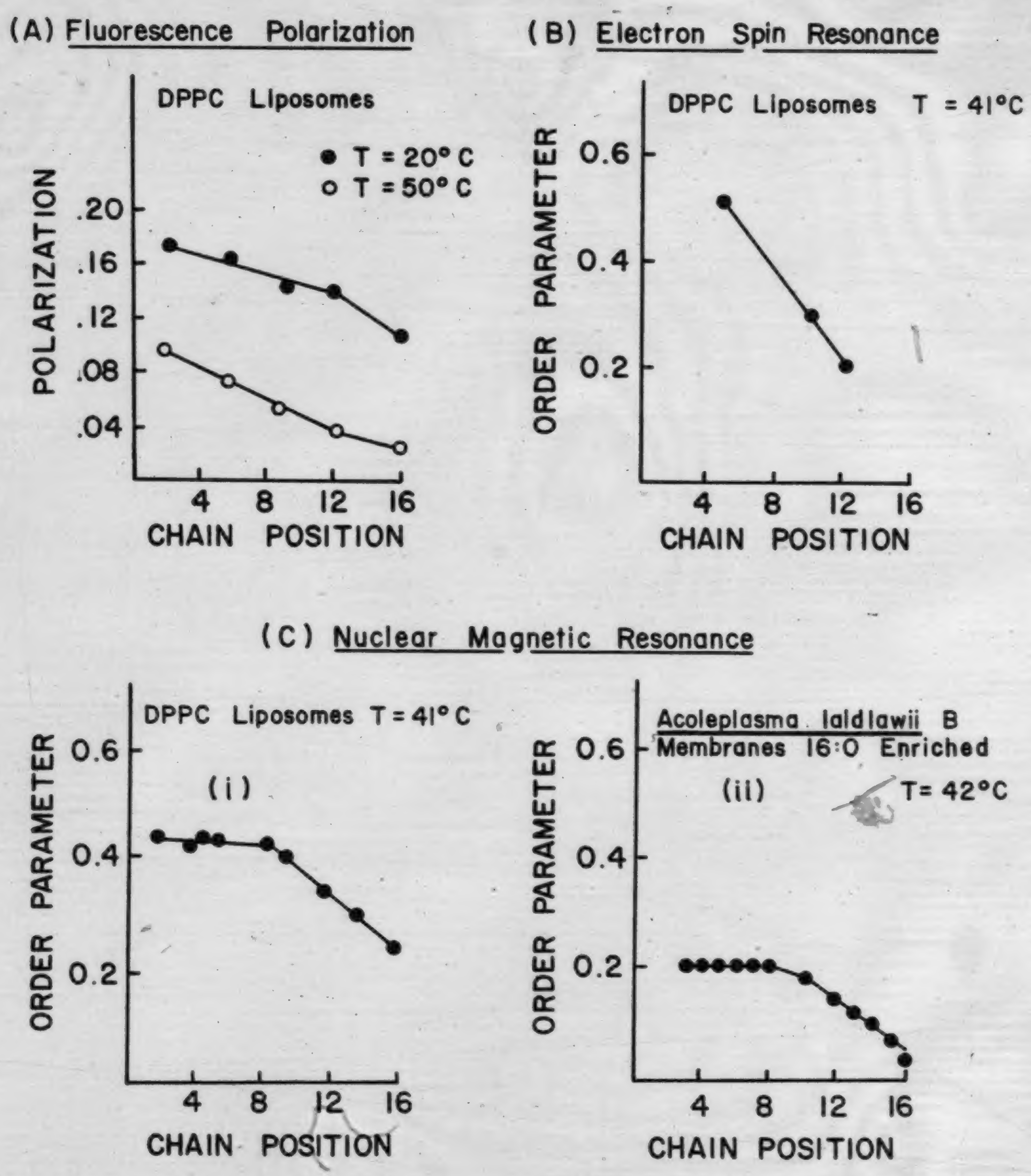


Fig. 2. The order profiles of lipid bilayers obtain with fluorescence, ESR, and NMR spectroscopy. For references see text.

distances between the hyperfine splittings an order parameter may be calculated (Anderson, 1978). In addition, the width of the resonance lines are sensitive to the rates of motion within the time scale of 10^{-7} to 10^{-9} seconds. Motions with frequencies of less than 10^{7} hertz (Hz) are too slow to be detected by ESR, while those greater than 10^{9} Hz are isotropically averaged to zero. As is the case for fluorescent probes, the ESR probes are very sensitive, thus only small quantities of the probe are needed, typically of the order of one percent.

The similarities between the ESR and fluorescent probes outlined above subject these probes to the same criticisms in terms of the amount of perturbation introduced into the bilayer. As late as 1975 some workers had concluded that ESR probes did not introduce any major perturbations into the bilayer at concentrations of less than or equal to 1% of the lipid present. It was thought these levels were low enough to preclude any perturbation due to the probe (Hubbell and McConnell,1971; Heminga, 1975). These conclusions were based on observations of little or no macroscopic changes in the properties of the lipid bilayer in response to the presence of the nitroxide probes. Nitroxide-labelled stearic acid intercalated into dipalmitoyl PC liposomes accurately reported the gel-to-liquid crystalline phase transition, seen as an abrupt increase in the mobility of the probe (Hubbell and McConnell, 1971). In addition, varying this probe concentration from 1% to as low as 0.02% did not affect the calculated values of the order parameter or the observed rates of motion of the probe (Heminga, 1975). These experiments, however, cannot answer questions about the microenvironmental changes introduced into the acyl chain properties by the probe molecules. Such changes could result in erroneous predictions concerning the absolute and relative values of both orientational and dynamical properties of the acyl chains. A comparison of the effects of the 12-nitroxide stearic acid and 12-(9-anthroyloxy) stearic acid in monomolecular films indicates that the nitroxide group is more disruptive than the anthroyloxy group. Significant increases in both the surface potential and the surface pressure of the film are seen when the nitroxide group is present (Cadenhead et al. , 1975). These results indicate the nitroxide moiety preferentially interacts with the aqueous surface at lower temperatures, due to the relatively high polarity of this group,which may result in its exclusion from the hydrophobic core when the bilayer is in the gel state. If the temperature is increased one observes an initial decrease in the surface pressure exhibited by the monolayer when the probe is present (Bell et al., 1978; Cadenhead and Muller-Landau, 1974). This is thought to occur because the increase in temperature decreases the order of the monolayer which allows the probe to intercalate into the hydrophobic region of the monolayer. This in turn would result in a reduction in the area occupied by the probe molecules. This conclusion is consistent with reports that 12-doxyl stearic acid preferentially partitions into the more fluid or disordered regions of the lipid bilayer (Butler et al., 1978; Oldfield et al., 1972). Comparison of the observed order parameter versus label position on the acyl chains of dipalmitoyl PC vesicles reveals large differences between the results obtained with NMR (using deuterated stearic acids) and ESR (using doxyl stearic acids) (Taylor and Smith, 1980; Seelig and Niederberger, 1974; Butler et al., 1978). The ESR probes indicate that the order of the acyl chains decreases linearly from the carbonyl group to the methyl terminus, whereas the NMR probes indicate that the order of the chains remains constant up to the 10-position, followed by a rapid decrease towards the methyl terminus (see fig. 2). These results are difficult to explain in the absence of any perturbation due to the ESR probe even in light of the time scale differences between the two techniques. If a motion is fast enough to be averaged on the NMR time scale but not the ESR time scale, then the additional anisotropic motion seen in the ESR experiment would be expected to increase the magnitude of the observed order parameters relative to those observed in the NMR experiment. In fact one sees a decrease rather than an increase in these values, indicating that these ESR probes introduce a significant perturbation into their microenvironment when incorporated into the hydrophobic region of the lipid bilayer (Seelig and Niederberger, 1974).

D. Nuclear Magnetic Resonance Spectroscopy

The disadvantages of NMR, that have restricted its use as a technique for the study of acyl chain order, are the relative insensitivity of this method and the complexity involved in trying to analyze the spectra once obtained. Recent technical advances have partially overcome the first problem, while the theoretical treatment required for the analysis of the spectra has become better developed in recent years (Peterson and Chan, 1977; Bloom *et al.*, 1975; Niederberger and Seelig, 1976).

A major advantage over the other spectral techniques is the non-perturbing nature of the NMR probes. A number of nuclei have proven useful in studies of model and biological membrane systems, notably: ^{1}H; ^{19}F; ^{2}H; ^{13}C; ^{31}P; and ^{15}N, listed in order of decreasing sensitivity with respect to the NMR experiment.

The nuclei ^{31}P and ^{15}N have been used to study the orientation and mobility of the lipid headgroups (Kohler and Klein, 1977; DuFourcq and Lussan, 1972), while ^{13}C has been shown to be potentially ideal for measurements of correlation times of both the headgroups and different regions of the acyl chains (Cornell et al., 1980). However, up to very recently the low sensitivity (.25 that of ^{1}H) coupled with the low natural abundance of ^{13}C (1.2%), has limited its use as a membrane probe (Smith, 1979).

Of the nuclei listed above primarily three, ^{1}H, ^{19}F, and ^{2}H, have been used to study the organization of acyl chains in a lipid bilayer. Of these ^{1}H is the most sensitive; however, it is ubiquitous in lipids, precluding its use for measurements of segmental order parameters along the acyl chain. Measurements of the average order of the chains expressed in terms of an order parameter have been determined (Ulmius et al., 1975), but this offers no insight into the differences that exist in the ordering of different regions of the acyl chains.

By far the most used nucleus in these types of studies has been deuterium (^{2}H). Fatty acids may be specifically deuterated so that the location of the probe in the bilayer is known, and the order of specific regions of the chains may be determined. The first study utilizing this technique was done with selectively deuterated dipalmitoyl PC with the deuterium located at various positions along the acyl chain (Seelig and Seelig, 1974). It was shown that the ordering of the palmitoyl chains above the phase transition remained constant up to 10 carbons from the glycerol backbone followed by a rapid decrease in the order from the 10-position to the methyl terminus (see fig. 2). The first application of the technique to a biological membrane was the incorporation of deuterated palmitic acids into the plasma membrane of Acholeplasma laidlawii B (Stockton et al., 1975). The isolated membrane contained approximately 70% palmitate, and the order parameter profile of this membrane above its phase transition temperature was remarkably similar to that observed in the dipalmitoyl PC model system, the only difference being a large decrease in the absolute values of the order parameter in the A. laidlawii B membrane, indicating that this biological membrane is much more disordered than the model systems. The success of these experiments has led to this technique being applied to other biological systems, notably Escherichia coli (Nichol et al., 1980; Davis etal., 1979). The disadvantage of deuterium is its relatively low sensitivity (0.41 that of ^1H) in the NMR experiment. In order to obtain spectra with signal-to-noise ratios comparable to those obtained with fluorine under similar experimental conditions, approximately 4 times the amount of deterium would be required. Thus large amounts of the probe are usually required in the system under study, and typically deuterated fatty acid enrichments of greater than 50 mole % are employed.

The greater sensitivity of fluorine (0.94 of ^1H) means that much less of the probe must be present, thus one can determine the order of the membrane bilayer in a system where the probe is not the major constituent of the membrane fatty acids. The similarity in size and reactivity of the fluorine atom in an alkyl chain to that of hydrogen suggests that fluorine may be a relatively non-perturbing probe of membrane structure. To date there have been studies done using monofluorinated fatty acid probes (Birdsall et al., 1971), as well as studies using difluorinated fatty acids, with the two fluorines bonded to the same carbon atom in the alkyl chain (Gent et al., 1976; Gent et al., 1978; Gent and Ho, 1978). All of the work in biological systems have been done with difluorinated fatty acids in Escherichia coli membranes; however, recent reports indicate that the difluorinated probe may introduce a significant perturbation into the acyl chain packing and interactions (Oldfield et al., 1980; Sturtevant et al. , 1979; Longmuir et al., 1977). DSC studies of dimyristoyl PC mixtures of the difluorinated and non-fluorinated myristic acids indicate that the two fluorine atoms may be acting as a "pseudo-unsaturated site in the acyl chain" (Sturtevant et al., 1979). In addition, observations of the order parameter from deuterium NMR in the presence and the absence of the difluorinated probe indicated that this substitution introduces a local disordering of the acyl chains, which was observed as a 30% decrease in the calculated deuterium order parameter in the region of the difluoro group (Oldfield et al., 1980).

In summary, each of the techniques discussed above have some limitations. Clearly the most versatile are the fluorescent and ESR probes, which require the presence of very small amounts of probe. However, one must always be aware of the perturbation introduced into the system when using these probes. The NMR probes are much less sensitive than either the fluorescent or ESR probes, which makes them impractical to use unless relatively large amounts of the probe may be introduced into the system under study. Nevertheless, the non-perturbing nature of these probes make them ideally suited for studies of the acyl chain order, where even small perturbations may lead to erroneous results.

The purpose of this work is to look at the effect of acyl chain structure on the order of fatty acid chains through the lipid bilayer in the membrane of A. laidlawii B, using a series of monofluorinated palmitic acid analogs as NMR probes. The approach used is to incorporate a number of structurally different fatty acids into the plasma membrane of A. laidlawii B along with a small amount of one of the fluorinated fatty acids. When this organism is cultured in the presence of avidin, a biotin-binding protein, endogenous fatty acid synthesis is abolished (Silvius and McElhaney, 1978a). Under these conditions the organism readily incorporates exogeneous fatty acids into its plasma membrane, thus the membrane may be made homogeneous with respect to its fatty acid composition for a number of different fatty acids (Silvius et al., 1980). If a small amount of one of the monofluorinated palmitic acid probes is added along with a particular exogenous fatty acid, then in principle the order parameter profile characteristic of that exogenous fatty acid may be determined.

With this in mind I have synthesized a series of monofluorinated palmitic acid analogs to use as NMR probes in this system. Fluorine was chosen because of its high sensitivity in the NMR experiment as well as its similarity in size and reactivity to hydrogen in an alkyl chain, with the expectation that this substitution will introduce very little perturbation into the bilayer. To test this assumption I have also carried out experiments using a number of different approaches to evaluate the magnitude of the perturbation induced by the substitution of a single hydrogen by one fluorine atom in a fatty acid chain.

II. MATERIALS AND METHODS

A. Materials

Growth Medium for Acholeplasma laidlawii B: Bacto peptone, bacto heart infusion broth and bacto yeast extract were obtained from Difco Laboratories (Detroit, Mich.). Fatty acid-depleted bovine serum albumin, a product of Miles Biochemicals (Elkhart, Ind.), was further delipidated following the procedure described by Chen (1967). Avidin was supplied by Sigma (St. Louis, Mo.) and penicillen G was obtained from Ayerst Laboratories (Montreal, Quebec).

Chemicals: All fatty acids used in this study were products of either Nu-Chek prep (Elysian, Minn.) or Analabs (North Haven, Conn.). Dipalmitoyl phosphatidylcholine (PC) and glycerophosphorylcholine cadmium chloride adduct, a precursor used in the synthesis of the PC's, were products of Sigma (St. Louis, Mo.). The precursors used in the synthesis of the monofluorinated palmitic acid derivatives were products of either Aldrich Chemicals (Milwaukee, Wisc.), Fischer Scientific (Edmonton, Alta.) or K and K (Irvine, Calif.). Tetrabutylammonium iodide and silver monofluoride, used in the synthesis of tetrabutylammonium fluoride, were products of Aldridge and ICN Pharmaceuticals, Inc. (Plainview, New York), respectively. All chemicals used were reagent grade. The organic solvents used were all redistilled and anhydrous solvents were prepared as described in Vogel (1956). All gases were products of Union Carbide of Canada (Oakville, Ont.). Inorganic reagents used in chemical synthesis and buffers were products of Fischer Scientific or Baker Chemical Co. (Phillipsburg, N.S.) and were of reagent grade or higher purity.

Chromatography Supplies: Gas-liquid chromatography (GLC) columns composed of 10% diethylene glycol succinate on Anakron B were products of Analabs. Bio-Sil A, used for column chromatography, was a product of Bio-Rad (Mississauga, Ont.). Silica gels G and H, used in analytical and preparative thin-layer chromatography (TLC), were products of Macherey, Nagel and Company (Germany).

Organism: *Acholeplasma laidlawii* B was originally obtained from Dr. G. Edwards (Wellcome Research Laboratories, Beckenham, England).

B. Methods

Growth Conditions: *Acholeplasma laidlawii* B was grown statically at 36°C in Razin media prepared as described by Silvius and McElhaney (1978b). 12 grams of bacto heart infusion broth together with 5 grams each of bacto peptone and bacto yeast extract were dissolved in 200 ml of distilled water, acidified to pH 4 with HCl and extracted twice with 50 ml of chloroform. The solution was made up to one liter by addition of distilled water, 3.7 grams of Tris-HCl was added as a buffer, and the solution was titrated to pH 8.2 with 40% NaOH. The media was autoclaved and stored at 4°C (up to three months) until used. The day before innoculation the media was supplemented with 0.4% weight by volume (w/v) lipid-depleted bovine serum albumin, 0.25% w/v glucose and 10^5 I.U./liter of penicillen G. 2.5 mg/liter of avidin was also added where stated. Exogeneous fatty acids were added to the growth media to a final concentration of 0.12mM. The fatty acid mixtures were first dissolved in 0.2ml of ethanol before added to the media.

Preparation of Membranes: Membranes were prepared as described by Pollack and co-workers (1965). Cells were harvested in late log phase by centrifugation for 20 minutes at 10,000 g. The cells from one liter of media were washed once in a solution of 0.154 M NaCl, 20 mMβ-mercaptoethanol and 0.05 M Tris-HCl at pH 7.4 (β-buffer). The cells were resuspended in 5 ml of β-buffer, diluted into 290 ml of distilled water at 37°C, and incubated for 30 minutes, resulting in cell lysis. Unlysed cells and cellular debris were pelleted at 8000 g for 8 minutes. The supernatant was then centrifuged at 30,000 g for 40 minutes to pellet the plasma membranes. The membrane pellet was washed once with 1/20 β-buffer then resuspended in one ml of 1/20 β-buffer containing 95% deuterium oxide for nuclear magnetic resonance (NMR) or DSC studies.

Lipid Extraction: Lipids were extracted from whole cells or isolated membranes following basically the procedure described by Bligh and Dyer (1959). Washed whole cells or cell membranes were pelleted and resuspended in 8 ml of a solution of 0.154 M NaCl, 0.01 M $MgSO_4$ and 0.02 M Tris-HCl at pH 8.0 (α-buffer). To this was added 20 ml of methanol and the solution was incubated at 50° for 30 minutes to solubilize the lipids and denature the proteins. To this solution 30 ml of chloroform ($CHCl_3$), 25 ml of water, then 50 ml CHCl were added sequentially with vigorous shaking after each addition. The upper aqueous phase was discarded and the $CHCl_3$ fraction was applied to a silicic acid column. The column was washed with four volumes of $CHCl_3$ to remove neutral lipids and caratenoids, and the eluant discarded. The polar lipid fraction was eluted from the column with methanol and concentrated *in vacuo*.

Fatty Acid Analysis: The fatty acid composition of the total membrane lipids was determined by GLC as described by Silvius and McElhaney (1978b). Isolated lipids were dissolved in anhydrous methanol (up to 10 mg/ml) containing 3% sulfuric acid and allowed to react for two hours at 60°C. Three volumes of water were added and the fatty acid methyl esters were extracted into two volumes of hexane. The samples were concentrated under nitrogen, dissolved in a small volume of hexane (100 ul), and analyzed by GLC, using a Hewlett-Packard 5700A gas chromatograph. The signal from the ionization detector which is proportional to the mass of the eluting compound, was coupled to a Hewlett-Packard 3370 B integrator for analysis.

Differential Scanning Calorimetry: DSC samples were prepared by vortexing known amounts of lipids with distilled water at 50-60°C for three minutes. DSC was performed with a Micro-Cal MC-1 high-sensitivity differential scanning calorimeter using a heating scan rate of 30°C/hour. The areas of the peaks were determined by tracing the peaks, cutting the traces out and weighing the paper. Each peak was done at least three times and the area taken as the average value obtained. These values were calibrated with the amount of heat absorbed. The final concentration of phospholipid in the samples was determined by quantitating the amount of esterified fatty acid present using GLC in the presence of a fatty acid internal standard.

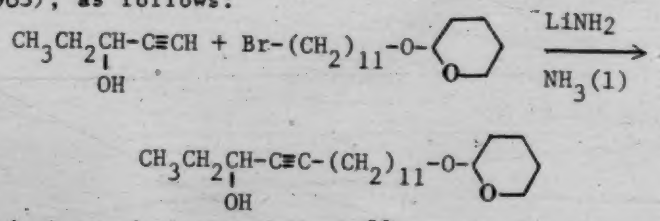
Nuclear Magnetic Resonance: Samples of membranes for NMR analysis were suspended in 1/20 β-buffer containing 95% deuterium oxide as previously described. Spectra were collected using a Bruker-270 MHz spectrometer at a fluorine resonance frequency of 254.025 MHz, using quadrature detection at a spectral width of ±50,000 Hz. Where spectra were collected at different temperatures for the same sample, the temperature was allowed to equilibrate for 30 minutes before spectra were collected. Temperatures were accurate to 1°K. Typically $2.5\text{x}10^4$ scans were accumulated for samples in which the membrane lipids were in the liquid-crystalline state (2 hrs), and 1.25x10^5 scans when the gel state (8 hrs). Samples were pulsed for 15 usec followed by a 20 usec receiver blanking pulse. An acquisition time of 200 msec was used and the free induction decay (FID) was digitized into 4 K data points. The FID's were collected in blocks of 5000 scans which were averaged together to give the final block-averaged FID, which was stored on a computer disc for analysis. Where the first two or three points of the FID were scrambled by pulse breakthrough, they were linearly extrapolated to zero time before fourier transformation of the FID. Under these conditions the spectra obtained required only zero-order phase corrections to give the final spectra with good baselines. Typically, signal-to-noise ratios of 30 to 1 were obtained when a line broadening of 50 Hz was applied to the FID's. Spectra collected at 188.217 and 376.333 MHz were obtained using similar experimental conditions.

C. Synthesis of Fluorinated Lipids

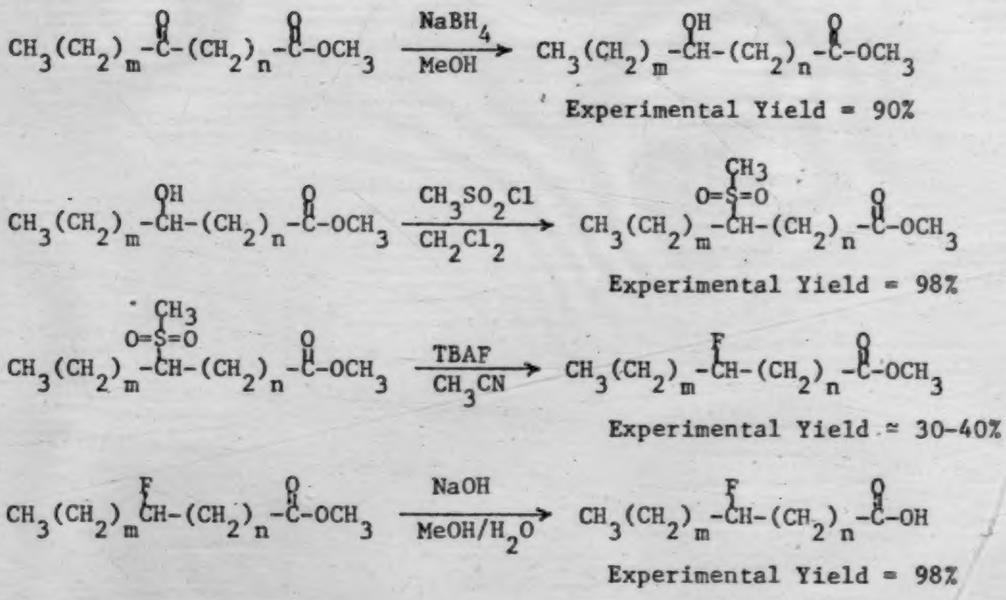
I. Fluorinated Fatty Acids:

Mono-fluorinated fatty acids of the form $CH_3(CH_2)_m$-CHF-$(CH_2)_n$-COOH where $m+n=13$ were synthesized from the corresponding keto-acid methyl esters following a modified procedure of Birdsall and co-workers (1971). The keto acid methyl ester was selectively reduced to the hydroxy acid methyl ester with $NaBH_4$. The product (yield 90%) was purified by silicic acid column chromatography and concentrated *in vacuo*. The hydroxy derivative was mesylated using methane sulfonyl chloride in the presence of triethyl amine, and the mesylated fatty acid methyl ester was extracted into CH_2Cl_2, concentrated *in vacuo*, and used without further purification (yield=98%). This product was dissolved in dry acetonitrile in the presence of five equivalents of dry tetrabutylammonium fluoride and reacted for three days at room temperature under anhydrous conditions. This reaction yields 30-40% of a saturated monofluorinated fatty acid methyl ester. The unsaturated by-product form was oxidized following the procedure of Von Rudloff (Christie, 1973), and the monofluorinated fatty acid methyl ester was isolated by acid-base extraction, concentrated *in vacuo* and crystallized twice, once from acetone and once from hexane, at -20°C. The purity was estimated to be greater than 99% by GLC and analytical TLC. The methyl ester was hydrolyzed to the free fatty acid in distilled methanol in the presence of 0.1 normal KOH (Silvius and McElhaney, 1978b). The chemical synthesis of monofluorinated palmitic acids is summarized in Figure 3.

The synthesis of the keto-acid methyl esters followed exactly the procedure of Hubbell and McConnell (1971) for the 5-,8-,10-, and 12-fluorinated species. The precursor required for the synthesis of the 14-keto-acid methyl ester via this pathway were not soluble in the reaction solvents, so an alternate method was used for this analog. The lithio anion of the ethyl ethylnylcarbinol dissolved in liquid ammonia was coupled to 11-Bromo-undecanol in which the primary alcohol was protected with dihydropyran in the presence of a mild acid catalyst (Ame and Covell, 1963), as follows:



This product was hydrogenated at 50 pounds per square inch (PSI) of hydrogen gas in the presence of platinium oxide as a catalyst, deprotected in methanol in the presence of an acid catalyst, and



$$CH_3(CH_2)_m\text{-}\overset{O}{\overset{\|}{C}}\text{-}(CH_2)_n\text{-}\overset{O}{\overset{\|}{C}}\text{-}OCH_3 \xrightarrow[MeOH]{NaBH_4} CH_3(CH_2)_m\text{-}\overset{OH}{\overset{|}{C}}H\text{-}(CH_2)_n\text{-}\overset{O}{\overset{\|}{C}}\text{-}OCH_3$$

Experimental Yield = 90%

$$CH_3(CH_2)_m\text{-}\overset{OH}{\overset{|}{C}}H\text{-}(CH_2)_n\text{-}\overset{O}{\overset{\|}{C}}\text{-}OCH_3 \xrightarrow[CH_2Cl_2]{CH_3SO_2Cl} CH_3(CH_2)_m\text{-}\overset{O=\overset{CH_3}{\overset{|}{S}}=O}{\overset{|}{C}}H\text{-}(CH_2)_n\text{-}\overset{O}{\overset{\|}{C}}\text{-}OCH_3$$

Experimental Yield = 98%

$$CH_3(CH_2)_m\text{-}\overset{O=\overset{CH_3}{\overset{|}{S}}=O}{\overset{|}{C}}H\text{-}(CH_2)_n\text{-}\overset{O}{\overset{\|}{C}}\text{-}OCH_3 \xrightarrow[CH_3CN]{TBAF} CH_3(CH_2)_m\text{-}\overset{F}{\overset{|}{C}}H\text{-}(CH_2)_n\text{-}\overset{O}{\overset{\|}{C}}\text{-}OCH_3$$

Experimental Yield ≈ 30-40%

$$CH_3(CH_2)_m\overset{F}{\overset{|}{C}}H\text{-}(CH_2)_n\text{-}\overset{O}{\overset{\|}{C}}\text{-}OCH_3 \xrightarrow[MeOH/H_2O]{NaOH} CH_3(CH_2)_m\text{-}\overset{F}{\overset{|}{C}}H\text{-}(CH_2)_n\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH$$

Experimental Yield = 98%

Fig. 3. The synthetic pathway used to synthesize the monofluoropalmitic acid derivatives from the corresponding keto-acid methyl esters.

oxidized to 14-keto palmitic acid using three equivalents of $K_2Cr_2O_7$ dissolved in acetone at 0°C.

II. Synthesis of Fluorinated Phosphatidylcholines:

The di-monofluoropalmitoyl phosphatidylcholines (PC's) were synthesized according to the procedure of Patel *et al.* (1979). The fatty acid was reacted with dicyclohexylcarbodiimide in dry carbon tetrachloride overnight at room temperature to produce the fatty acid anhydride. This was reacted with the cadmium chloride adduct of glycerophosphorycholine in an equal volume solution of dry benzene and dimethylsulfoxide in the presence of 4-pyrolidinylpyridine, which acts as a catalyst. The products were purified by silicic acid column chromatography to remove free fatty acid, and preparative TLC using silica gel G and the solvent system 50:50:4 methanol: chloroform: ammonium hydroxide to remove the lyso-phosphatidylcholine present. The isolated PC was greater than 99% pure as determined by GLC and analytical TLC.

D. Theory of Lineshape Analysis

For a fluorine atom in an alkyl chain undergoing anisotropic motion, the relaxation has contributions from both dipolar and chemical shift anisotropic (CSA) mechanisms. If it is assumed that these relaxation mechanisms are independent, then it is possible to derive expressions for each that will describe their contribution to the observed lineshape.

The chemical shift (σ) of a spin undergoing anisotropic motion is defined by

$$\sigma=\sigma_i+2/3\ \sigma_a\left(\frac{3\cos^2\theta-1}{2}\right) \qquad (1)$$

where σ_i is the isotropic chemical shift and

$\sigma_a = (\sigma_{11} S_{11} + \sigma_{22} S_{22} + \sigma_{33} S_{33})$.

For a lipid bilayer the motion of the lipid molecules is axially symmetric or $\sigma_{22} = \sigma_{33}$, and σ_a may be expressed in terms of one independent order parameter and a constant, $(\sigma_{\parallel} - \sigma_{\perp})$, which defines the maximal difference in the chemical shift due to the CSA, or,

$$\sigma_a = (\sigma_{\parallel} - \sigma_{\perp}) S_z \tag{2}$$

In this case, the observed resonance frequency, ν, is defined as:

$$\nu = \nu_i - 2/3(\sigma_{\parallel} - \sigma_{\perp}) S_z \nu_o \left(\frac{3\cos^2\theta - 1}{2} \right) \tag{3}$$

where ν_i is the isotropic frequency and ν_o is the instrumental fluorine frequency. The angle θ is defined as the angle the acyl chains long axis makes with respect to the axis of symmetry in the bilayer, that being the bilayer normal.

For large vesicles (>>250 Å), where the tumbling rate is slow with respect to the NMR time scale, the orientation of the bilayer will be randomly distributed with respect to the applied magnetic field. In this case a powder type spectrum will be observed where for each value of σ there corresponds an unique resonance line at ν. A probability function $p(\nu)$ may now be defined such that $p(\nu)d\nu$ is the probability of a spin having a resonance frequency between ν and $\nu + d\nu$.

$$p(\nu) = \left[1 - \frac{2(\nu - \nu_i)}{2/3(\sigma_{\parallel} - \sigma_{\perp}) S_z \nu_o} \right]^{-1/2} \tag{4}$$

For dipolar relaxation the spin hamiltonian is time dependent because of molecular motion$\langle H_d\rangle(t)$ may be separated into a time-independent term and a time-dependent term (Bloom et al.,1975)

$$H_d(t)=\langle H_d\rangle+[H_d(t)-\langle H_d\rangle] \qquad (5)$$

For all cases where $\langle H_d\rangle= 0$ the line shape contribution of d(t) will be dominated by $\langle H_d\rangle$. In the case of large vesicles (>>250 A in diameter), $\langle H_d\rangle = 0$ and therefore the experimental lineshape will be governed by $\langle H_d\rangle$. This term is composed of an angularly independent part (interdipole interactions), $\langle H_d\rangle_i$, and an angularly dependent part (interdipole interactions), $\langle H_d\rangle_\theta$, or: $\langle H_d\rangle=\langle H_d\rangle_i+\langle H_d\rangle_\theta$

$$\langle H_d\rangle_\theta=\langle H_d\rangle_o\left(\frac{3\cos^2\theta-1}{2}\right) \qquad (6)$$

if one defines $\Delta=\langle H_d\rangle$, $\Delta_o=\langle H_d\rangle_i$, $\Delta_1=\langle H_d\rangle_\theta$

then $$\Delta=\Delta_o+\Delta_1\left(\frac{3\cos^2\theta-1}{2}\right) \qquad (7)$$

If it is assumed that the dipolar contribution is averaged by the same molecular motions as the CSA then we can say

$$\Delta=\Delta_o+\Delta_1\left(\frac{3\cos^2\theta-1}{2}\right)S_z \qquad (8)$$

If each resonance line, ν , has a transition probability $I(\nu)$ and each resonance line centered at ν_i is approximated by a normalized Gaussian, then

$$I(\nu-\nu')= \frac{1}{2\pi}\ \frac{1}{\Delta} \exp\left[\frac{-(\nu-\nu')^2}{2\Delta^2}\right] \qquad (9)$$

The observed lineshape $S(\nu)$ is described by $I(\nu-\nu')$ weighted by the probability of finding a resonance line at ν defined by $p(\nu)$ over all possible orientations (Niederberger and Seelig, 1976).

$$S(\nu)=\int_{-\infty}^{\infty} I(\nu-\nu')\ p(\nu')\ d\nu' \qquad (10)$$

A computer program has been written (see Appendix 1) to calculate theoretical lineshapes according to eq. 10, which allows for the estimation of the order parameter, Sz.

III. EVALUATION OF THE USEFULNESS OF MONOFLUORINATED PALMITIC ACIDS AS NMR PROBES OF MEMBRANE STRUCTURE

A. Introduction

Fluorine was chosen as an NMR probe because of its high sensitivity in the NMR experiment and its similarity in size and reactivity to hydrogen in an alkyl chain. This atom may also be incorporated into specific positions of an acyl chain following established chemical reaction pathways (see Materials and Methods for details). Thus these probes offer the possibility of being both very sensitive and specific probes of acyl chain organization in lipid bilayers. The first question to be addressed is the amount of perturbation introduced into the acyl chain order by the presence of the fluorine atom. In the case of the difluorinated fatty acids, the assumption that substitution of two hydrogen atoms with two fluorine atoms is an innocuous change has recently been called in to question (Sturtevant *et al.*, 1979; Oldfield *et al.*, 1980). These workers' results indicate the difluoro group exhibits some properties of a site of unsaturation in the acyl chain. This observation agrees with NMR experiments in which the order parameter, measured with specifically deuterated fatty acids, decreased by approximately 30% in the region of the difluoro group compared to when this group is not present. Thus it appears that this probe disrupts the local acyl chain packing; however, this perturbation is much less than that seen with other spectral probes such as ESR or fluorescent probes.

In order to evaluate the effect of the monofluorinated fatty acids I have taken three approaches. Firstly, I have carried out studies, similar to those done with the difluorinated fatty acids by Sturtevant and co-workers (1979), to compare the thermotropic behavior of di-monofluoropalmitoyl PC with those of dipalmitoyl PC, using high-sensitivity DSC. Secondly, the effects the biosynthetic incorporation of various quantities of these probes on the growth of *A. laidlawii* B, as well as the ability of this organism to distinguish biochemically between fluorinated and non-fluorinated palmitic acids, have been investigated. Finally, using ^{19}F-NMR spectroscopy, I have determined the order parameters, using these monofluorinated probes, of the plasma membrane lipids of this organism, enriched with palmitic acid, in order to compare these values to the previously reported order parameters obtained for this system by ^{2}H-NMR using specifically deuterated palmitic acids (Stockton *et al.*, 1977). I have also varied the levels of these probes to determine the dependence, if any, of the measured order parameter on the concentration of the probe in these palmitic acid-enriched membranes.

B. *Results and Discussions*

Thermotropic behavior of di-monofluoropalmitoyl PC's and mixtures of these and dipalmitoyl PC.

The di-monofluoropalmitoyl PC's were synthesized and samples of these were prepared for DSC studies as described in Materials and Methods. Three fluorinated analogs were used in these studies, with the fluorine atom located at the 5-, 8-, or 14- position of the fatty acid chain. The temperatures of the main transition (Tm) and the pre-transition (Tp) for the di-monofluoropalmitoyl PC's are listed in Table 1. In each case the Tm is lower than that observed for dipalmitoyl PC. The

Table 1

The thermotropic phase transition properties of the pure di-monofluoropalmitoyl PC's.

Lipid	Tm^{o}C	Tp^{o}C	ΔHcal $\frac{Kcal}{mole}$	ΔHvH $\frac{Kcal}{mole}$	C U S $\frac{\Delta HvH}{\Delta Hcal}$
5,5-di-monofluoropalmitoyl PC	34.9	29.1	5.8±0.25	160±8	28
8,8-di-monofluoropalmitoyl PC	39.6	22.1	6.5±0.3	380±19	58
14,14-di-monofluoropalmitoyl PC	40.1	29.1	8.8±0.35	300±15	34
dipalmitoyl PC	40.3	35.0	8.3±0.3	900±45	108

largest decrease is seen when the fluorine atom is in the 5-position of the acyl chain, where the Tm was 5.4° lower than the Tm of dipalmitoyl PC. The decrease in the Tm's for the 8- and 14-di-monofluoropalmitoyl PC's were 0.7° and 0.2° respectively, indicating the fluorine atom perturbation decreases as the position of the substitution moves toward the methyl terminus of the chain. The corresponding transition curves are shown in figure 4. The transition curves of the fluorinated lipids are broader than those of dipalmitoyl PC, indicating that the presence of the fluorine atom decreases the cooperativity of the transition. In the case of a lipid bilayer the cooperative unit size (CUS) is defined as the number of lipid molecules acting together as a single unit when the bilayer undergoes a thermotropic transition such as from the gel to the liquid-crystalline state (Stankowski and Gruenwald, 1980). The CUS is empirically defined as the ratio of the Van't Hoff enthalpy ($\Delta H_V H$), which is a measure of the sharpness of the transition (Mabrey and Sturtevant, 1978), over the calorimetric enthalpy (ΔHcal), which is a measure of the heat absorbed by the molecules due to the transition. The CUS determined for the fluorinated lipids are listed in Table 1. Each of these lipids exhibits a decrease in their CUS as compared to the non-fluorinated species, with the greatest effect again observed when the fluorine atom is located in the 5-position. The cooperativity is least affected when the fluorine atom is towards the middle of the chain. These observations suggest the presence of the fluorine atom tends to disrupt somewhat the acyl chain interactions in the bilayer.

The transition curves also show there is a marked decrease in the Tp's in the fluorinated lipids, with the largest decrease (12°) observed when the fluorine atom is in the 8-position of the chain. The

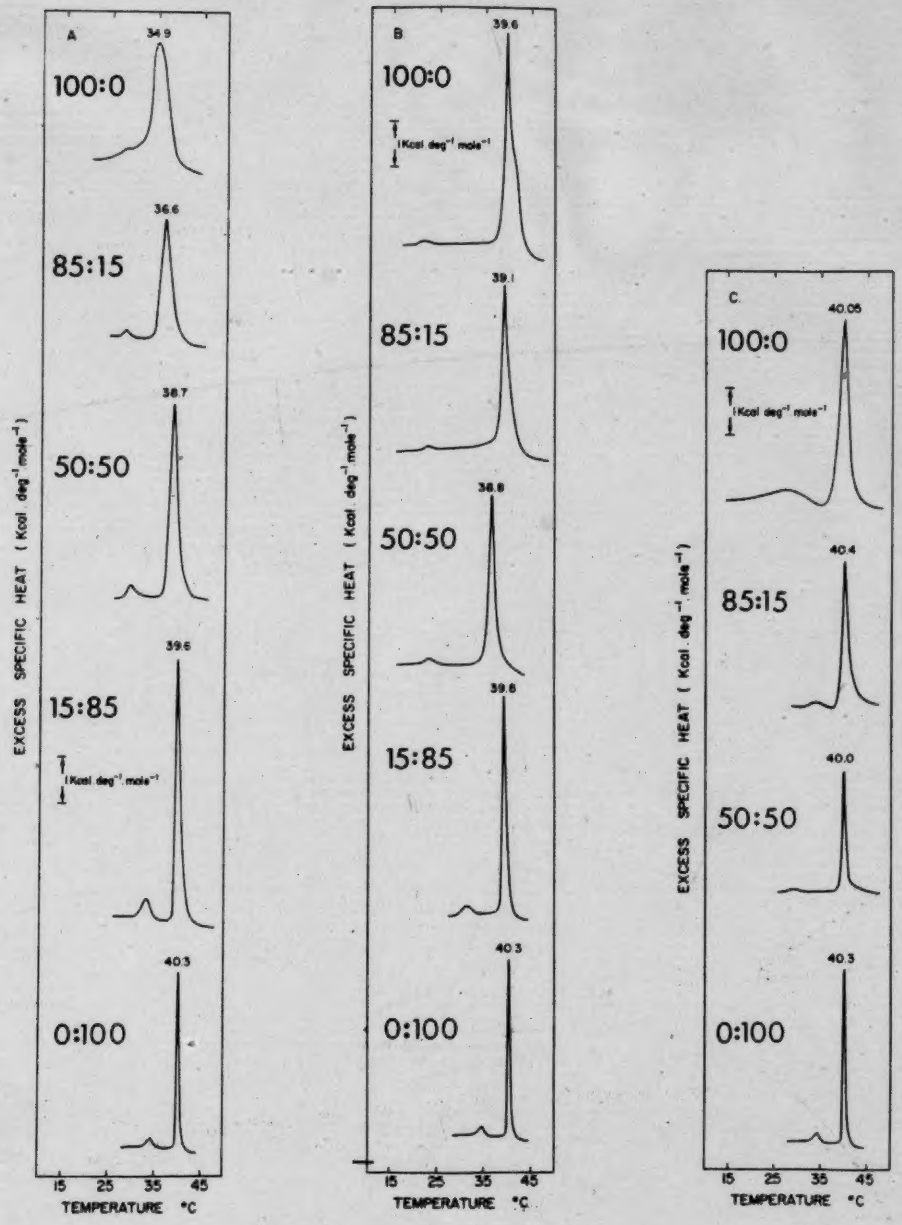


Fig. 4. The transition curves for the 5-(panel A), 8-(panel B), and 14-(panel C) di-monofluoropalmitoyl PC's and mixtures of these with dipalmitoyl PC. Ratios by weight of monofluoropalmitoyl PC to dipalmitoyl PC are given beside each curve.

pre-transition is thought to be associated with a structural transformation from a one dimensional lamellar, where the hydrocarbon chains are fully extended and tilted with respect to the bilayer normal, to a two dimensional lattice in which the lipid lamellae is distorted by a periodic ripple (Janiak et al., 1976). This conformation is stable between the Tp and the Tm temperatures of the lipid. The 5- and 14-fluorinated analogs each show a 6° decrease in the observed Tp's. However, the continued existence of the pre-transition, which is found only in relatively pure phospholipids containing straight-chain saturated fatty acids, is taken as evidence that these fluorinated analogs closely mimic the physical behavior of palmitic acid.

The results also show a change in the enthalpies of the main transitions of these lipids compared to dipalmitoyl PC. A decrease of 30% and 22% in the enthalpies of the 5- and 8-fluoro analogs, respectively, is observed, while in contrast the 14-fluoro analog had a slightly higher enthalpy value, approximately 6%. Comparison of these results with those obtained by Sturtevant and his co-workers (1979) for the difluoromyristoyl PC's suggests that the monofluorinated fatty acids introduce much less perturbation into the lipid bilayer. In the presence of the difluoro group, the enthalpy was found to increase by a factor of two, and the Tm's varied from the corresponding non-fluorinated lipid by +4.7° when the fluorine atoms were in the 4-position to -7.7° when they were in the 8-position of the 14-carbon chain.

An excellent method to determine the extent of perturbation introduced into the bilayer by the fatty acid probes is to examine the thermotropic behavior of mixtures of the fluorinated and non-fluorinated PC's. If the perturbation is minimal, then one would expect to see nearly ideal mixing of the lipids as well as transition curves similar to those observed for the pure lipids with respect to their shape and calculated enthalpies. The transition curves of various mixtures of each of the substituted lipids with dipalmitoyl PC are shown in figure 4. From these curves it is apparent that the lipids exhibit close to ideal mixing, with the major effect of the fluorine atom being a decrease in the cooperativity of the transitions. The Tm's of the di-5-monofluoropalmitoyl PC - dipalmitoyl PC mixtures lie between the Tm's of the two pure PC's. In contrast, for mixtures containing the di-8-monofluoropalmitoyl PC the Tm's are slightly lower than the value of the pure fluorinated species, whereas for the di-14-monofluoropalmitoyl PC mixtures essentially no change in the Tm values was observed. It is interesting to note the transition cure for the pure 8-fluoro analog exhibits a slight assymetric shoulder that disappears when the lipid is mixed with dipalmitoyl PC. In all cases the cooperativity increases in the presence of dipalmitoyl PC, indicating that no lateral phase separation of the two lipids is occurring in the mixtures. The transition properties calculated for these mixtures are listed in Table 2. In these mixtures one might expect to observe the largest perturbation effects when the ratio of the two lipids approaches one-to-one; however, at this ratio the Tm's and the enthalpies of the mixtures are approaching the values observed for the dipalmitoyl PC. The biggest differences are observed when the fluorine atom is in the 5-position, as before, but the difference in the enthalpy is less than 2 Kcal/mole. Under these conditions, when the fluorine atom is located in the 8-position , the enthalpy difference is less than 1 Kcal/mole, while no difference was seen when it was in the 14-position of the fatty acid chain. The pre-

Table 2

The thermotropic phase transition properties of mixtures of the di-monofluoropalmitoyl PC's and dipalmitoyl PC.

a) Mixtures containing 85% by weight of di-monofluoropalmitoyl PC and 15% by weight dipalmitoyl PC

Fluoro-lipid present in the mixture	Tm°C	Tp°C	ΔHcal $\frac{\text{Kcal}}{\text{mole}}$	ΔHvH $\frac{\text{Kcal}}{\text{mole}}$	C U S	$\frac{\Delta\text{HvH}}{\Delta\text{Hcal}}$
5,5-di-monofluoropalmitoyl PC	36.6	28.0	6.0±0.25	222±10	37	
8,8-di-monofluoropalmitoyl PC	39.1	23.4	6.9±0.3	476±24	69	
14,14-di-monofluoropalmitoyl PC	40.4	34.1	8.5±0.35	382±20	45	

b) Mixtures containing 50% by weight of di-monofluoropalmitoyl PC and dipalmitoyl PC

Fluoro-lipid present in the mixture	Tm°C	Tp°C	ΔHcal $\frac{\text{Kcal}}{\text{mole}}$	ΔHvH $\frac{\text{Kcal}}{\text{mole}}$	C U S	$\frac{\Delta\text{HvH}}{\Delta\text{Hcal}}$
5,5-di-monofluoropalmitoyl PC	38.7	28.4	6.5±0.3	358±18	55	
8,8-di-monofluoropalmitoyl PC	38.8	25.5	7.4±0.35	555±28	75	
14,14-di-monofluoropalmitoyl PC	40.0	29.7	8.3±0.35	564±28	68	

transition is present in all of the mixtures, as one would expect if the presence of the fluorine results in only minor perturbations of the bilayer. If we again compare these observations to those obtained with the difluorinated derivatives, it may be concluded that the monofluorinated acids are much less perturbing. In the case of the difluorinated acids, large deviations in the enthalpies and the Tm's, as well as the existence of lateral phase separations of the difluorinated and the corresponding non-fluorinated PC's, were observed when similar mixing studies were done (Sturtevant *et al.*, 1979).

Growth Studies with *Acholeplasma laidlawii* B

Cells were cultured in lipid-depleted media supplemented with various ratios of palmitic acid and monofluorinated palmitic acids in the absence of avidin, following the procedure described in Materials and Methods. The total amount of 16:0 fatty acid incorporated into the plasma membrane lipids remained constant at approximately 75 to 80% of the total fatty acid present (see Table 3). As was the case with the DSC experiments, the fluorinated fatty acids used in these studies were primarily the 5-,8-, and 14-fluorinated analogs. The results show that the growth of this organism is unaffected until the fluoropalmitate exceeds 30% of the total palmitate present, where the total palmitate corresponds to the fluoropalmitic acid plus the palmitic acid present (see fig. 5). Above this amount one observes a progressive decrease in the cell yield as determined by the absorbance of the culture at 450 nm against a suitable blank (McElhaney and Tourtelotte, 1970). The largest effect was seen when the fluorine atom was in the 5-position of the fatty acid chain, with the magnitude of the decrease in cell yield becoming progressively smaller as the fluorine atom position moved

Table 3

The fatty acid composition of A. laidlawii B membranes from cells cultured in media supplemented with 85% palmitic acid and 15% of one of the mono-fluorinated palmitic acid analogs.

Fatty acid supplement	Fatty Acids in membrane (%)						
	12:0	14:0	16:0	Fluoro-16:0	18:0	18:1	n.d.*
16:0 (control)	2.1	12.8	77.5	--	2.4	1.0	3.5
5-Fluoro 16:0	2.2	9.8	68.5	10.8	2.8	0.8	5.1
8-Fluoro 16:0	2.1	11.4	66.7	9.6	2.0	1.0	8.1
10-Fluoro 16:0	3.1	10.4	65.7	10.4	2.1	1.9	7.5
12-Fluoro 16:0	2.0	11.6	66.4	9.9	2.4	1.2	5.6
14-Fluoro 16:0	2.0	12.0	65.1	11.0	2.1	1.7	5.1

Values determined by GLC

*n.d. Not determined, in this case no single fatty acid represented more than 1% of the total fatty acid present.

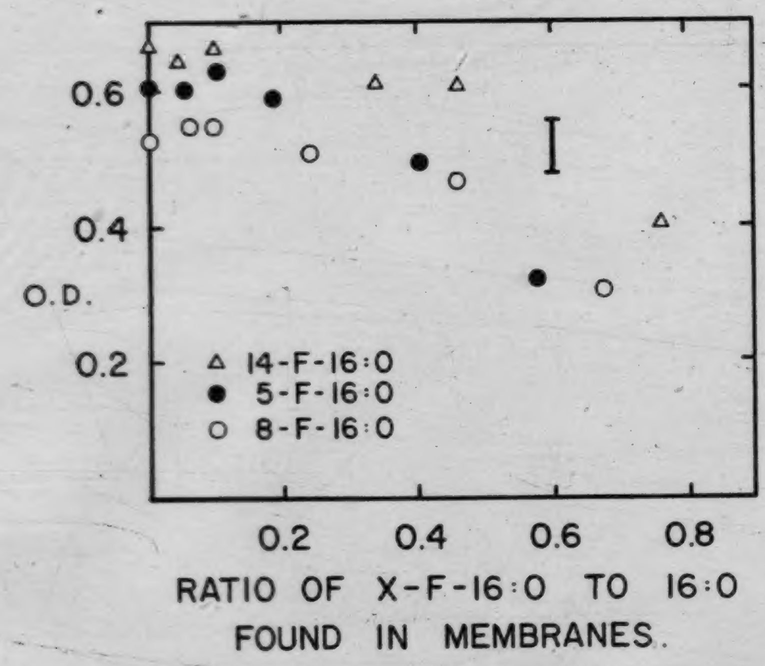


Fig. 5. The effect of increasing amounts of monofluoropalmitic acid incorporated into the membrane of A. laidlawii B enriched with palmitic acid on the cell yield.

toward the methyl terminus of the chain. It was also found that the ratio of the fluoropalmitic acid to palmitic acid incorporated into the membrane lipids corresponded to the ratio of these fatty acids added to the growth media, indicative that the organism does not biochemically distinguish between the monofluorinated and non-fluorinated palmitic acids.

Palmitic acid cannot support growth of these cells in the presence of avidin, a potent inhibitor of de novo fatty acid biosynthesis (Silvius and McElhaney, 1978b); however, a variety of structurally different fatty acids can support excellent growth in the presence of avidin, resulting in plasma membranes homogeneous with respect to their fatty acid composition (Silvius and McElhaney, 1978b). In light of these observations, I have grown cells in the presence of avidin in media supplemented with a monofluorinated palmitic acid (or palmitic acid as a control), together with a second fatty acid capable of supporting good growth when added alone. The results obtained for a variety of different fatty acids indicate that under these conditions the presence of the fluorine atom had little or no effect on cell yield up to ratios of one-to-one of the fluorinated palmitic acid to the other exogenous fatty acid (fig. 6). In addition, the level of incorporation of the fluoropalmitate in each case corresponded to the level of incorporation of palmitic acid into the membrane in the control experiments.

Nuclear Magnetic Resonance Experiments

Samples were prepared as described in Materials and Methods. The first approach was to determine the orientational order parameter profile of A. laidlawii B membranes enriched with palmitic acid (70-80% palmitate) containing approximately 10% of one of the fluoropalmitate

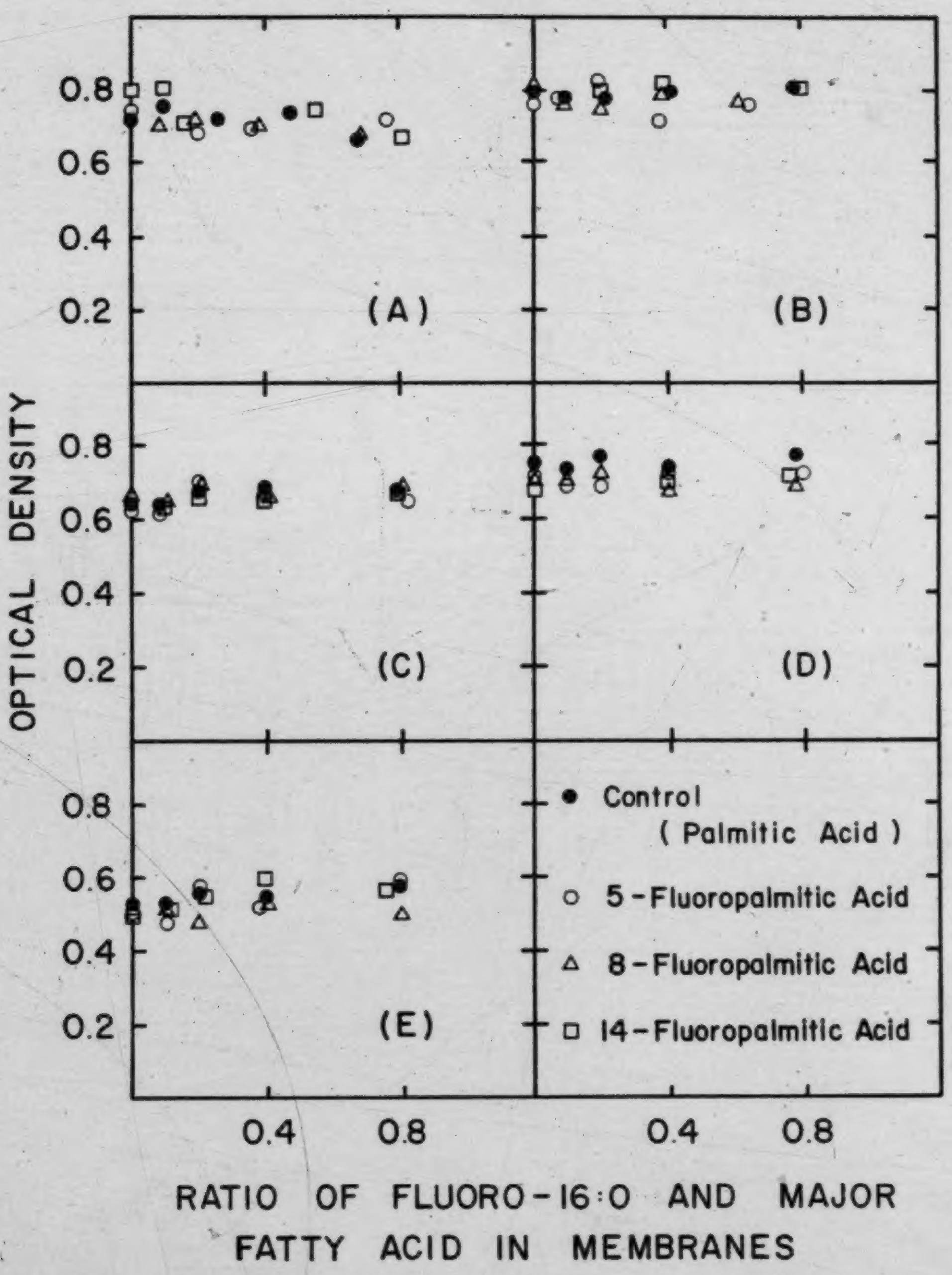


Fig. 6. The effect of increasing amounts of monofluoropalmitic acid incorporated into the membrane of *A. laidlawii* B enriched with (a)16:1tΔ', (B) 18:1tΔ', (C) 16:0i, (D) 16:0ai, and (E) 15:0. For the control pamitic acid was used instead of one of the monofluoropalmitic acids.

probes. Table 3 lists the fatty acid composition of the membranes enriched with these fatty acids. These experiments were done to compare the order parameters obtained with ^{19}F-NMR to those obtained with ^{2}H-NNR in the same system in which the membrane lipids were enriched with specifically deuterated palmitic acid probes (Stockton *et al.* 1977). The results obtained with the different probes are presented in fig. 7. The order parameter profiles of these membranes are similar, with the major difference observed in the absolute values of the order parameters. In the plateau region, from carbons 2 to 10 of the acyl chain, the ^{19}F-NMR-determined order parameter is less than those observed with ^{2}H-NMR, with values of 0.18 and 0.22, respectively. Beyond the 10-position the values converge, with the values for the 14-position with both probes being the same within experimental error. The deuterium order parameters were derived from the quadrapolar splittings due solely to the lipids in the liquid-crystalline state at 42°C. At this temperature a small portion of the lipid is in the gel state, but the gel lipid only decreases the intensity of the signal of the liquid-crystalline lipid and not the quadrapole splitting from which the order parameters are derived. The fluorine order parameters were obtained at 45°C where essentially all of the membrane lipid is in the liquid-crystalline state (see fig. 8). This figure also shows that the phase transition curve for palmitate-enriched membranes containing a one-to-one ratio of fluoropalmitate and palmitic acid is essentially identical to that of palmitate-enriched membranes that have no fluoropalmitate present. The ^{19}F-NMR order parameters were determined by a curve fitting procedure, therefore the observed value includes a contribution from the gel state lipid if any is present. The differences observed in the values determined by the two probes may be

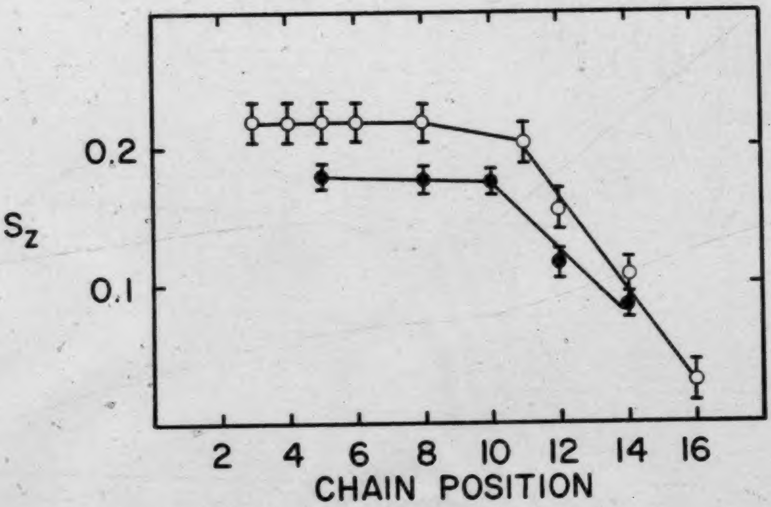


Fig. 7. The order parameter profiles obtained with specifically deuterated palmitic acids, (open circles) taken from (Stockton et al.,1977) and monofluorinated palmitic acids (closed circles) incorporated into palmitic acid-enriched membranes of A. laidlawii B

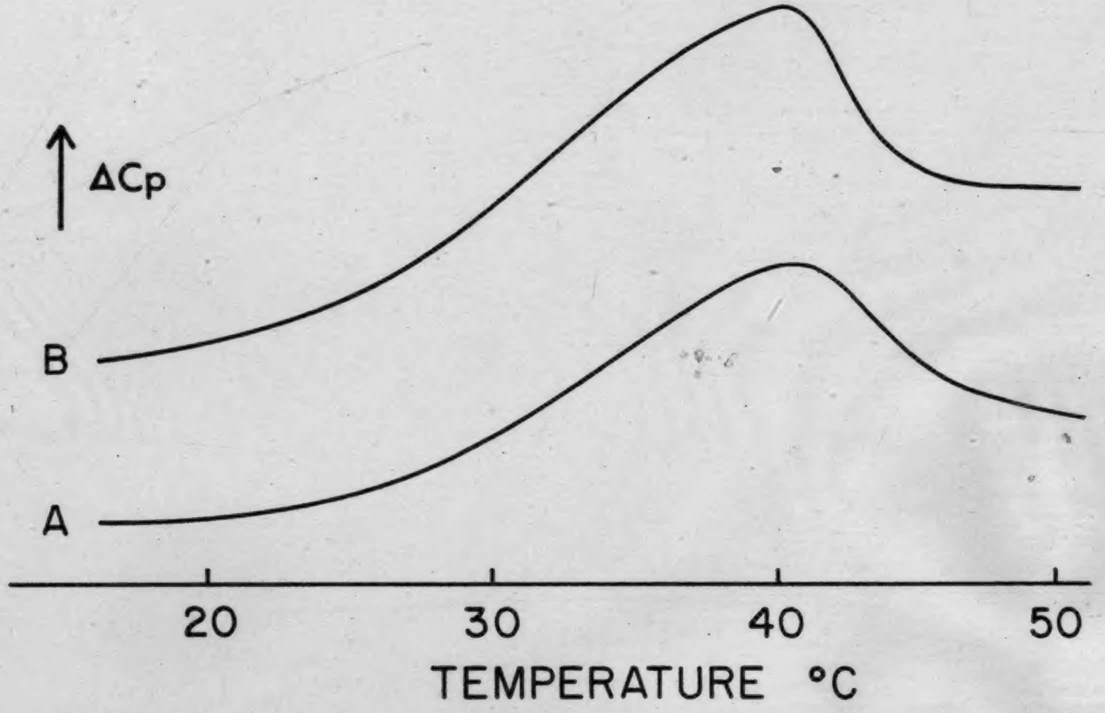


Fig. 8. Phase transition curves of palmitic acid-enriched membranes from A. laidlawii B cells. Top membranes containing 40 mole % monofluoropalmitic acid. Bottom membranes containing only palmitic acid.

because the presence of the fluorine atom results in a slight decrease in the order of the acyl chains in the region of the fluorine atom. This effect has been reported for the difluorinated fatty acids, where the local order is decreased by approximately 30% around the difluoro group (Oldfield et al., 1980). A second possible reason for the difference in the two sets of values may be the temperature difference in the two experiments. The fluorine spectra were collected at 45°C, three degrees higher than the deuterium spectra. Thus one might expect the liquid-crystalline lipid to have slightly more thermal energy, resulting in a small decrease in the order; however, a three degree difference does not appear to be enough to explain this difference in the plateau region, where small changes in temperature within the region of the liquid-crystalline state does not result in significant changes in the observed order parameter values. An example of this is seen in fig. 25 in which 18:1t enriched membranes have identical order parameter profiles at 34^0 and 37^0C. The fact that the largest absolute difference in the two sets of values occurs closest to the headgroup, whereas the values observed for the positions near the methyl terminus are more nearly the same, is consistent with previous results indicating that the fluorine atom is most perturbing when it is substituted into the 5-position of the fatty acid chain. However, the relative change in the order parameter determined from each nucleus remains constant for all positions along the chain.

Overall, the two probes are in excellent qualitative agreement in reporting the order parameter profile through the bilayer. There is also good quantitative agreement between the different techniques in terms of the absolute values of the order observed. The difference that is observed indicates that the presence of the fluorine atom near the polar headgroup results in a relatively small perturbation of the acyl chain order, expressed as a decrease in the observed order parameter for this region of the membrane bilayer.

A second approach undertaken was to determine if the order parameter was influenced by the amount of fluorinated palmitate present in palmitic acid-enriched membranes. One would not expect the ratio of fluoropalmitate to palmitic acid in these membranes to affect the observed values of the order. The results in figure 9 indicate this to be the case for the 5- and 14-fluorinated analogs of palmitate above the phase transition (45°C), in the region of the phase transition (37°), and below the phase transition (20°) (see Appendix B for the corresponding spectra). Within the error of the experiment, the observed order parameters remain constant for membranes containing from 5% to 50% of the fluorinated probe.

C. Conclusions

Taking the results presented in this chapter together with the results observed with the difluorinated fatty acids, the picture that emerges is that the monofluorinated fatty acids introduce only a small perturbation into the acyl chain order of a lipid bilayer. This perturbation is markedly less than that caused by the difluorinated species as seen by DSC and NMR experiments. In all of the studies that were done, the greatest effect was observed when the fluorine substitution was near the carboxyl group of the fatty acid. When the substitution was located close to the methyl terminus, very little or no difference was exhibited as compared to the case when the fluorine atom was not present.

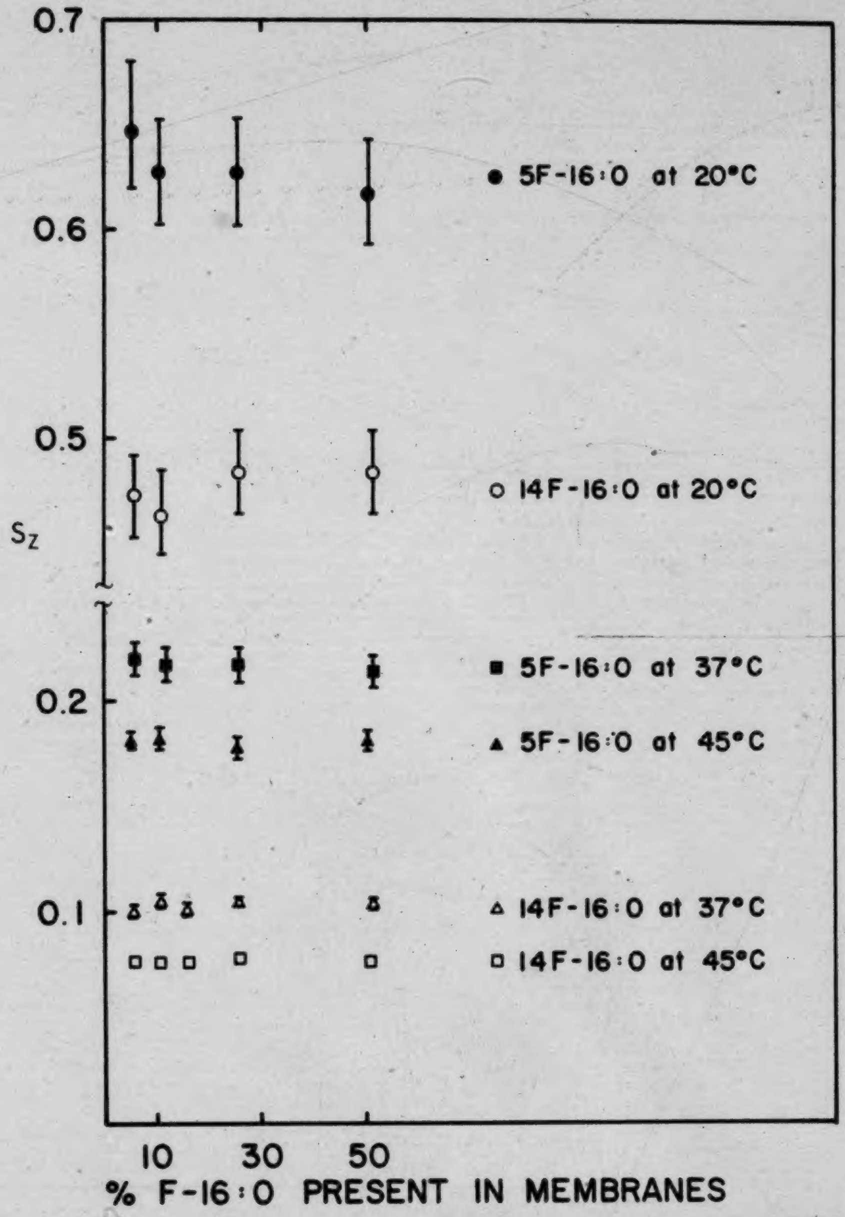


Fig. 9. The dependence of the order parameter on the concentration of either 5-or 14-monofluoropalmitic acid in palmitic acid-enriched membranes above and below the phase transition (45° and 20°, respectively) and at the growth temperature of 37°C.

The ease with which the fatty acid composition of the plasma membrane of A. laidlawii B may be manipulated to produce membranes containing predominantly one fatty acid, together with a small amount of one of the fluoropalmitic acid probes, affords the opportunity to determine the qualitative and quantitative effects of a number of structurally different fatty acids on the order profile and the absolute values of the order parameter in a biological membrane, keeping in mind the presence of some perturbation due to the presence of the monofluorinated palmitic acid.

IV. THE EFFECT OF FATTY ACID STRUCTURE AND TEMPERATURE ON THE ORDER OF THE ACYL CHAINS IN ACHOLEPLASMA LAIDLAWII B MEMBRANES AS SEEN WITH ^{19}F-NMR

A. Introduction

Membranes contain a number of structurally different fatty acids. These differences include variation in the chain length, from as short as twelve carbons to twenty carbons or more, differences in the number and location of double bonds, and in some cases the presence of a methyl branch near the methyl terminus of the fatty acid chain. There are some clues as to the function of these different fatty acid structures; for example, it has been observed that the phase state of lipids in membranes influence the activity of proteins and the transport of metabolites across membranes (Fox and Keith, 1972). It has also been reported that the activity of some membrane-bound enzymes is dependent on the presence of *cis*-double bonds (Rothfield and Romeo, 1971). In addition, it is known that acyl chain structure plays a crucial role in maintaining the membrane bilayer in a physiologically active state by modulating membrane fluidity (Overath *et al.*, 1975; McElhaney, 1974). Despite these observations, the role of the numerous different lipid classes, as well as the fatty acid heterogeneity present in biological membranes, is not well understood. Only a few fatty acid classes have been studied in terms of their effects on the orientational organization of acyl chains in lipid bilayers. The most extensively studied have been the straight-chain saturated fatty acids, in particular 16:0 (Davis, 1979; Seelig and Seelig, 1974; Stockton *et al.*, 1977). The order parameter profiles of this fatty acid in both PC bilayers and biological membranes are similar (see fig. 2), indicating that PC bilayers are good model systems for biological membranes. The effect of a cis-double bond on the order of acyl chains in a bilayer has also been studied (Seelig and Seelig, 1977). In contrast to the straight-chain saturated fatty acids, which show a plateau region from the 2 position to the 10 position of the chain, the presence of the cis-double bond causes a local increase in the order of the neighbouring fatty acids in the region of the double bond. There is also an overall decrease in the hydrocarbon ordering throughout the bilayer. Thus changes in the fatty acid structure not only affect the absolute order of the bilayer, but also have marked effects on the relative order of different regions of the fatty acid chains. These observations have led us to consider what the effect of other structural features of fatty acids have on the hydrocarbon order in a biological membrane. To this end we have cultured A. laidlawii B cells in the presence of avidin to obtain fatty acid-homogeneous membranes containing a variety of structurally different fatty acids, along with a small amount of one of the monofluoropalmitic acids as a probe of the acyl chain order, with the hope of gaining some insight into the structure-function relationship of other fatty acids in membrane bilayers.

B. Results and Discussion

Fatty Acid Composition of the Membranes Used in these ^{19}F-NMR Studies.

The membrane samples for NMR studies were prepared according to the procedure described in Chapter 2. Aliquots of these samples were used to determine the fatty acid composition of the membrane lipids by

GLC. Five monofluorinated palmitic acids, in which the fluorine atom was substituted at the 5-,8-,10-,12-, or 14-position of the alkyl chain, were used as ^{19}F-NMR probes in these studies. Small amounts (8-11 mole %) of each of these probes were incorporated into the membrane of *A. laidlawii* B cells along with one of five different fatty acids capable of supporting good growth of this organism in the presence of avidin, namely pentadecanoic acid (15:0), 14-methyl-pentadecanoic acid (16:0i), 13-methyl-pentadecanoic acid (16:0ai), *trans*-9-hexadecanoic acid (16:1tΔ^9), and *trans*-9-octadecanoic acid (18:1tΔ^9). The fatty acid compositions of these membranes are listed in Tables 4 through 8. Avidin has been shown to almost completely inhibit fatty acid metabolism in this organism (Silvius and McElhaney, 1978b). Of the above listed fatty acids, four (16:0i, 16:0ai, 16:1tΔ^9, 18:1tΔ^9), together with the fluorinated probe, accounted for 96.7-99.6 mole % of the total fatty acid present in the membrane. The other fatty acids found most likely originate from the media which, although it is extensively de-lipidated, still contains small amounts of residual fatty acids; a small amount of endogenous fatty acid synthesis may also occur. Cells cultured in the presence of avidin and supplemented with 15:0 show slightly higher background levels of fatty acids. In these membranes, 15:0 and the fluorinated probe account for 93.3 to 96.0 mole % of the total fatty acid. The background fatty acids present are composed primarily of even-chain saturated fatty acids of the type synthesized by *A. laidlawii* B cells, suggesting that a slightly higher amount of endogenous fatty acid synthesis is occurring in these cells (see Table 8). However, levels of 18:1 are also increased, and since this organism cannot synthesize unsaturated fatty acids, it also appears the exogenous fatty acid

Table 4

Fatty Acid Composition from Membranes of Acholeplasma laidlawii B Cells Supplemented with 90% 16:0i and 10% Fluoro-16:0 and Grown in the Presence of Avidin.

	Fatty Acids in membrane (%)[a]				
Fatty acid supplement	16:0	Fluoro-16:0	16:0i	18:0	18:1
5-Fluoro 16:0+16:0i	0.6	10.0	87	1.2	1.1
8-Fluoro 16:0+16:0i	--	10.2	88	1.6	0.3
10-Fluoro 16:0+16:0i	0.7	9.9	86	1.9	1.6
12-Fluoro 16:0+16:0i	--	12.7	85	1.7	0.4
14-Fluoro 16:0+16:0i	0.3	10.6	87	1.5	0.7

[a] Where the fatty acids listed do not represent 100%, other minor fatty acids were present that were not identified. These accounted for less than 0.2% of the total fatty acids with no individual one at levels greater than 0.1%.

Table 5

Fatty Acid Composition from Membranes of *Acholeplasma laidlawii* B Cells Supplemented with 90% 16:0ai and 10% Fluoro-16:0 and Grown in the Presence of Avidin.

	Fatty Acids in membrane (%)[a]				
Fatty acid supplement	16:0	Fluoro-16:0	16:0ai	18:0	18:1
5-Fluoro 16:0+16:0ai	0.8	9.8	86	2.1	1.3
8-Fluoro 16:0+16:0ai	0.7	10.0	89	0.3	--
10-Fluoro 16:0+16:0ai	0.2	10.4	88	0.7	0.7
12-Fluoro 16:0+16:0ai	0.4	10.8	88	0.5	0.3
14-Fluoro 16:0+16:0ai	0.9	9.6	87	1.6	0.8

[a] See table 4 footnote

Table 6

Fatty Acid Composition from Membranes of Acholeplasma laidlawii B Cells Supplemented with 90% 16:1tΔ⁹ and 10% Fluoro-16:0 and Grown in the Presence of Avidin.

	Fatty Acids in membrane (%)[a]			
Fatty acid supplement	16:0	Fluoro-16:0	16:1tΔ⁹	18:0
5-Fluoro 16:0+16:1tΔ⁹	1.2	9.0	88	1.8
8-Fluoro 16:0+16:1tΔ⁹	--	10.0	88	1.8
10-Fluoro 16:0+16:1tΔ⁹	1.0	11.0	86	2.0
12-Fluoro 16:0+16:1tΔ⁹	1.0	10.0	87	2.0
14-Fluoro 16:0+16:1tΔ⁹	--	8.1	90	1.6

[a] See table 4 footnote

Table 7

Fatty Acid Composition from Membranes of *Acholeplasma laidlawii* B Cells Supplemented with 90% $18{:}1t\Delta^9$ and 10% Fluoro-16:0 and Grown in the Presence of Avidin.

Fatty acid supplement	Fatty Acids in membrane (%)[a] 16:0	Fluoro-16:0	$18{:}1t\Delta^9$	18:0
5-Fluoro $16{:}0+18{:}1t\Delta^9$	0.5	8.7	90	--
8-Fluoro $16{:}0+18{:}1t\Delta^9$	0.8	10.3	88	0.9
10-Fluoro $16{:}0+18{:}1t\Delta^9$	1.1	9.4	89	0.4
12-Fluoro $16{:}0+18{:}1t\Delta^9$	0.4	11.0	88	0.6
14-Fluoro $16{:}0+18{:}1t\Delta^9$	0.6	10.6	89	--

[a] See table 4 footnote

Table 8

Fatty Acid Composition from Membranes of Acholeplasma laidlawii B Cell Supplemented with 90% 15:0 and 10% Fluoro-16:0 and Grown in the Presence of Avidin.

Fatty acid supplement	Fatty Acids in membrane (%)[a]					
	12:0	15:0	16:0	Fluoro-16:0	18:0	18:1
5-Fluoro 16:0+15:0	0.6	87	1.7	8.5	0.2	2.0
8-Fluoro 16:0+15:0	0.2	87	1.6	8.7	0.9	0.7
10-Fluoro 16:0+15:0	1.0	84	1.0	9.7	1.0	0.8
12-Fluoro 16:0+15:0	0.5	83	1.3	11.0	1.1	2.4
14-Fluoro 16:0+15:0	0.4	88	1.4	8.0	0.8	1.2

[a] See table 4 footnote

incorporation is also elevated. Since cells supplemented with 16:0 will not grow in the presence of avidin, 15:0 represents the upper chain length limit of the straight-chain saturated fatty acids capable of supporting growth at 37°C (Silvius and McElhaney, 1978a). Thus growth in the presence of 15:0 may be suboptimal growth, and the cells may compensate by a small but increased amount of endogenous fatty acid synthesis, even in the presence of the inhibitor avidin. Nevertheless, in this case and the others, the major fatty acid (generally 86-91 mole %) in the membrane is the one of interest, and it appears that the order parameter values obtained by ^{19}F-NMR reflect primarily the characteristics of the major fatty acid present, rather than those of the monofluoropalmitic acid probes.

An Evaluation of the Methodology Used for the Determination of the Order Parameter.

The Materials and Methods section contains the theoretical derivation used to generate the theoretical lineshapes used to fit the observed spectra to obtain an order parameter value. This derivation is based on the work of Niederberger and Seelig (1976) and Gent and Ho (1978). There are four parameters to consider in fitting the theoretical lineshapes to the observed spectra, namely $(\sigma_{\|}-\sigma_{\perp})$, Δ_0 , Δ_1 , and Sz.

Although the chemical shift tensor for the CHF group has not been determined, the value of $(\sigma_{\|}-\sigma_{\perp})$, the maximum chemical shift anisotropy for the fluorinated acyl chain, assuming axial symmetry, may be approximated from model compounds, in this case teflon, to be 118 ppm (parts per million) (Mehring et al., 1971; Gent and Ho, 1978). This value of $(\sigma_{\|}-\sigma_{\perp})$ was used in the curve fitting program. Experimentally, it was observed that up to 20% changes in this value did not significantly change the observed Sz values, likely because the major effect of this parameter is on the extreme boundaries of the spectral line.

The second parameter, Δ_0 , represents the angularly independent dipolar relaxation, or in molecular terms, the inter-chain interaction of the fatty acids in the bilayer. For membrane lipids in the liquid-crystalline state, the translational diffusion of the lipids is fast enough to average these interactions to zero (Bloom et al., 1975). In the gel state this motion is no longer fast enough to completely average this motion, and Δ_0 takes on positive values measured in Hz. The effect of the development of positive Δ_0 values is an overall broadening of the lineshape, particularly in the region of the central spike of the spectrum (see fig. 10), with a progressively smaller effect in the regions more removed from the central spike. It is only this parameter that significantly affects this region of the lineshape, thus it is the broadness of the central spike in the experimental spectra that primarily determines the value of Δ_0 .

The third parameter listed, Δ_1 , is a measure of the angularly dependent dipolar relaxation or the intra-chain interaction of the fatty acids in the bilayer. This parameter is constant, but its effect on the lineshape is modulated by the value of Sz (see page 28 , equation 8). For small values of Sz, the term containing Δ_1 makes a relatively small contribution to the lineshape, whereas when Sz increases the effect of the Δ_1 term markedly increases. The value of Δ_1 was approximated by collecting spectra of identical samples at different magnetic field strengths. The chemical shift anisotropic relaxation component is dependent on the magnetic field strength, while the dipolar relaxation component is not. Therefore, as the field strength increases the

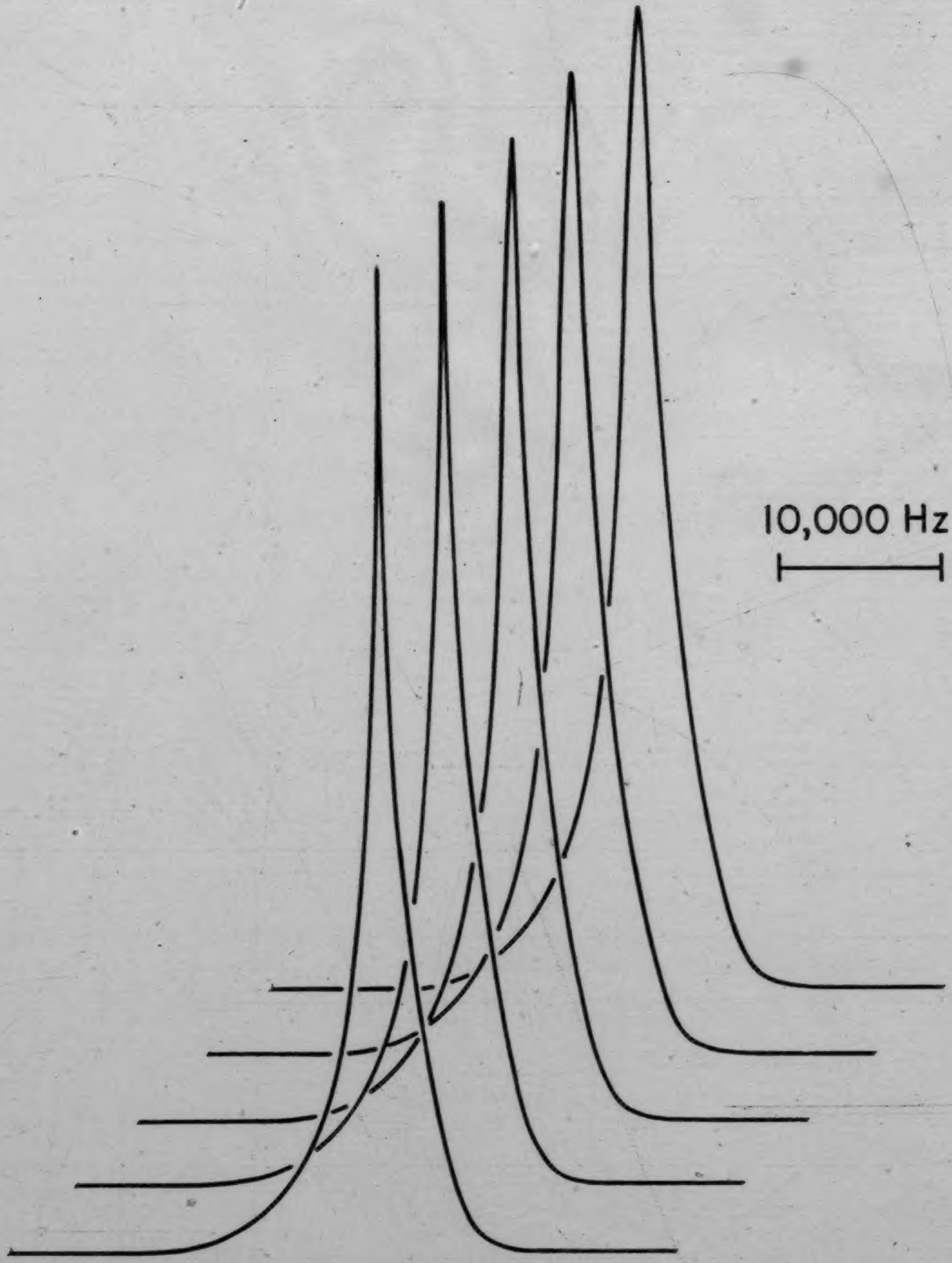


Fig. 10. The effect of changes of Δ_o on the theoretical lineshape of the calculated spectrum. Sz was set at 0.15 and Δ_1 was set at 20,000 Hz. From the bottom spectrum to the top spectrum the values of Δ_0 were 0, 50, 100, 150, and 200 Hz respectively.

spectral line will become broader, however the value of Δ_1 will remain constant. At a given temperature the value of Sz will also remain constant for a given membrane sample. Using these constraints I determined the best theoretical fits of spectra collected at 37°C and 45°C at field strengths of 188.217 and 254.025 MHz, from 16:0-enriched membranes containing 5-fluoropalmitic acid (see fig. 11), and temperatures of 20°C and 37°C at field strengths of 188.217, 254.025, and 376.333 MHz for membranes enriched with 16:1tΔ' containing 14-fluoropalmitic acid (see fig. 12). The values giving the best fit, using the above criteria, are given in Table 9. The best experimental spectra observed were those collected at 254.025 MHz followed by those collected at 188.217 MHz in terms of being able to phase the spectra and the signal-to-noise ratio obtained. With this in mind, a value of 20,000 Hz was selected for the value of Δ_1, which is biased towards the theoretical fits obtained at the two smaller field strengths. Changes of 10% in the value of Δ_1 result in comparable changes in Sz; however, this value was used in all subsequent lineshape analyses for determinations of Sz. Any error in this value would result in an overestimation or an underestimation of the order parameter values. This error, if present, is thought to be relatively small, because of the close similarity between the Sz values obtained using the deuterium and fluorine probes (see fig. 7). The variation in the values listed in Table 9 lie within the experimental error since slight differences in experimental temperatures and in the characteristics of the different NMR spectrometers used could have resulted in the small changes in the observed Δ_1 and Sz values.

The only adjustable parameters used to fit the observed spectra with the theoretical lineshape were Δ_0 and Sz. As previously mentioned,

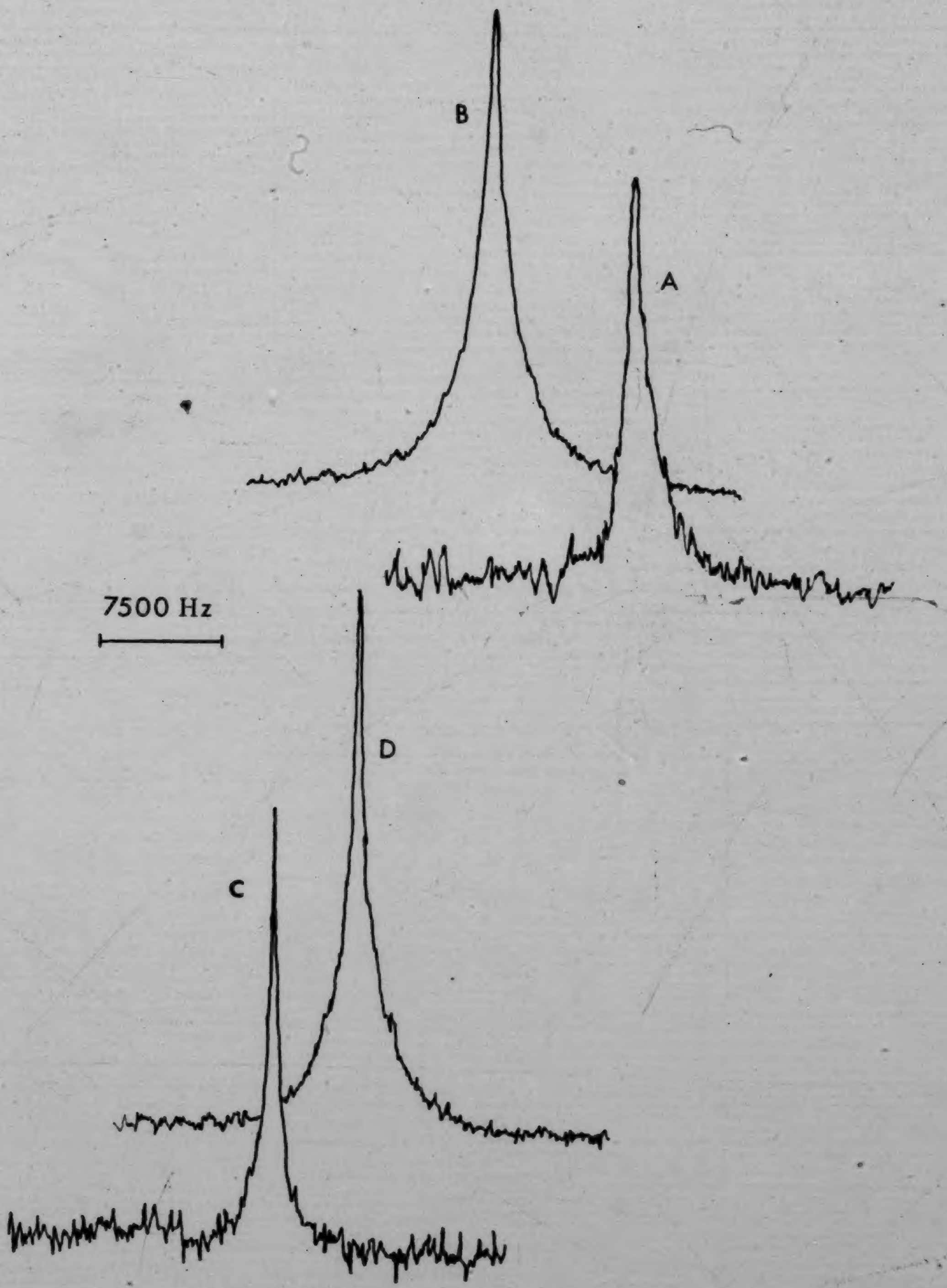


Fig. 11. The NMR spectra of membranes enriched with 16:0 containing 5-fluoropalmitic acid at field strengths of 188.217 MHz at 37°C (A) and 45°C (C) and at 254.025 MHz at 37°C (B) and 45°C (D).

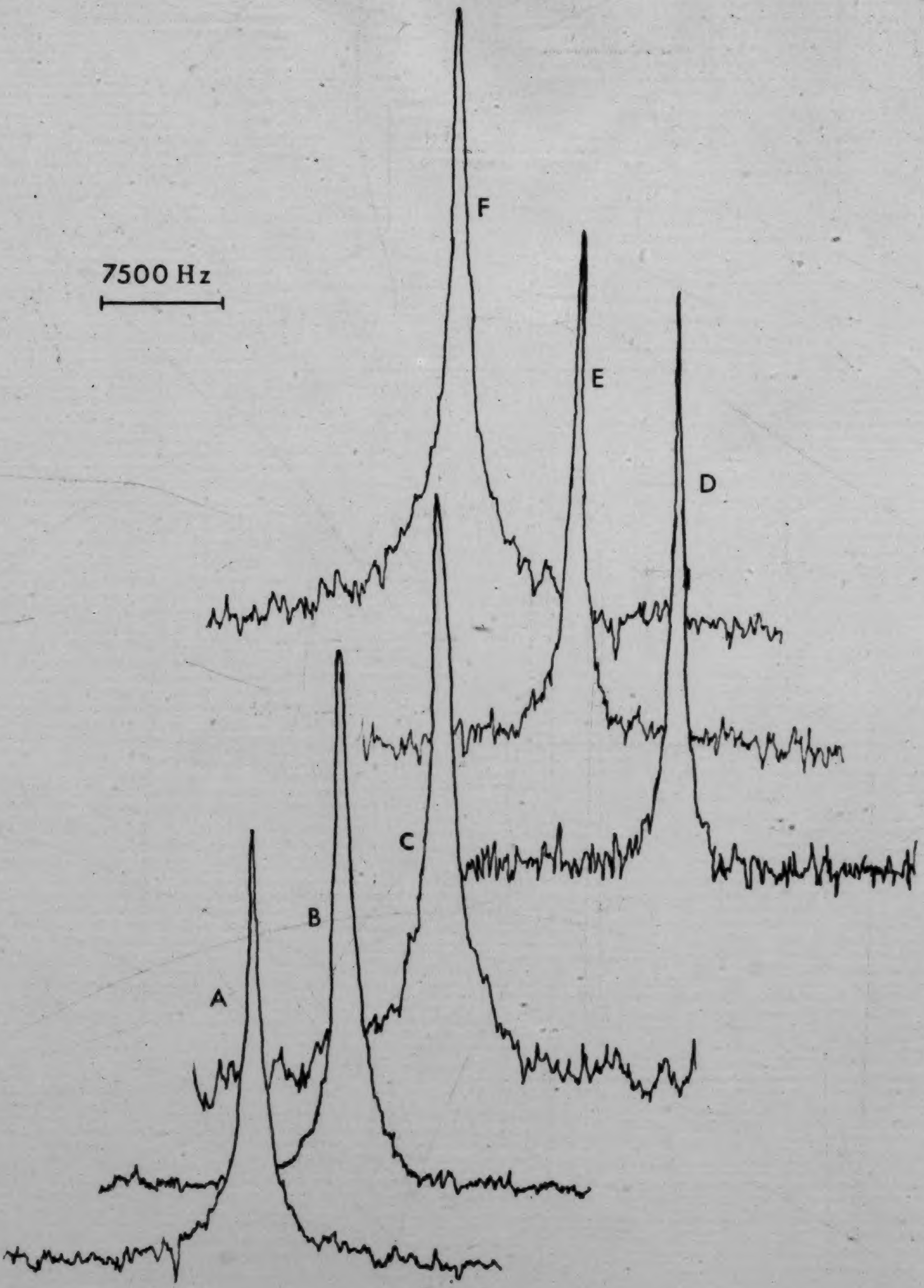


Fig. 12. The NMR spectra of membranes enriched with 16:1tΔ^9 containing 14-fluoropalmitic acid at field strengths of 188.217 MHz at 20° (A) and 37°C (D), at 254.025 MHz at 20° (B) and 37°C (E), and at 376.333 MHz at 20° (C) and 37° (F).

Table 9

The Best Parameters of Fit Determined for 16:1tΔ^9-and 16:0-enriched Membrane Samples at Different Field Strengths.

a) 16:1tΔ^9-enriched membranes containing 14-monofluoropalmitic acid as a probe

	Field Strength								
	188.217 MHz			254.025 MHz			376.333 MHz		
T°C	Δ_0	Δ_1	Sz	Δ_0	Δ_1	Sz	Δ_0	Δ_1	Sz
37	0	20,000	0.06	0	21,000	0.064	50	22,000	0.08
20	0	22,000	0.13	0	20,000	0.12	50	23,000	0.12

b) 16:0-enriched membranes containing 5-monofluoropalmitic acid as a probe

	Field Strength						
	188.217 MHz			254.025 MHz			376.333 MHz*
T°C	Δ_0	Δ_1	Sz	Δ_0	Δ_1	Sz	
45	0	20,000	0.164	0	20,000	0.176	n.d.
37	0	20,000	0.215	0	20,000	0.216	n.d.

---

*Some difficulty arose in trying to determine the values at 376.333 MHz due to the extreme broadness of the spectra. Spectra of the 5-monofluoropalmitic acid were not obtained while those of the 14-monofluoropalmitic acid-enriched membranes contained a residual broadening as exhibited by the increase in the Δ_0 values which likely resulted from field inhomogenaeity across the broad spectra.

the primary effect of changes in Δ_o is in the width of the central spike of the spectra (see fig. 10). In contrast, the major effect of changes in Sz are on the "wings" of the spectra, away from the region of the central spike (see fig. 13). The non-overlapping nature of the effects of these two parameters means that the value of each is unique and confined to a narrow range (± 10% or less) for each of the observed experimental spectra.

The Effect of Fatty Acid Structure and Temperature on the Order Parameter Profile of *Acholeplasma laidlawii* B Membranes.

The order parameter profiles obtained at the optimum growth temperature of 37°C for 16:0-enriched membranes isolated from cells cultured in the absence of avidin, and for 15:0-, 16:0i-, and 16:0ai-enriched membranes from cells cultured in the presence of avidin, are depicted in fig. 14. The order profiles show marked variation depending on the major fatty acid present in the membrane. The three fatty acids 15:0, 16:0i, and 16:0ai are each 15 carbons in length with 16:0i having a methyl group in the 14-position and 16:0ai a methyl group in the 13-position of the acyl chain. At a constant temperature the average order through the bilayer decreases in the order 16:0>15:0>16:0i>16:0ai. This observation agrees with the thermal transition midpoints (Tm) of these membranes, where 15:0-enriched membranes have a Tm=36.7° while 16:0i and 16:0ai have Tm values of 21.8° and 4.1°, respectively (Silvius and McElhaney, 1978a). These results also agree with the idea that "fluidizing" fatty acids, defined as those with relatively low melting points, are more disordered than fatty acids with relatively high melting points. Thus the absolute order is decreased in the presence of a methyl group near the methyl terminus.

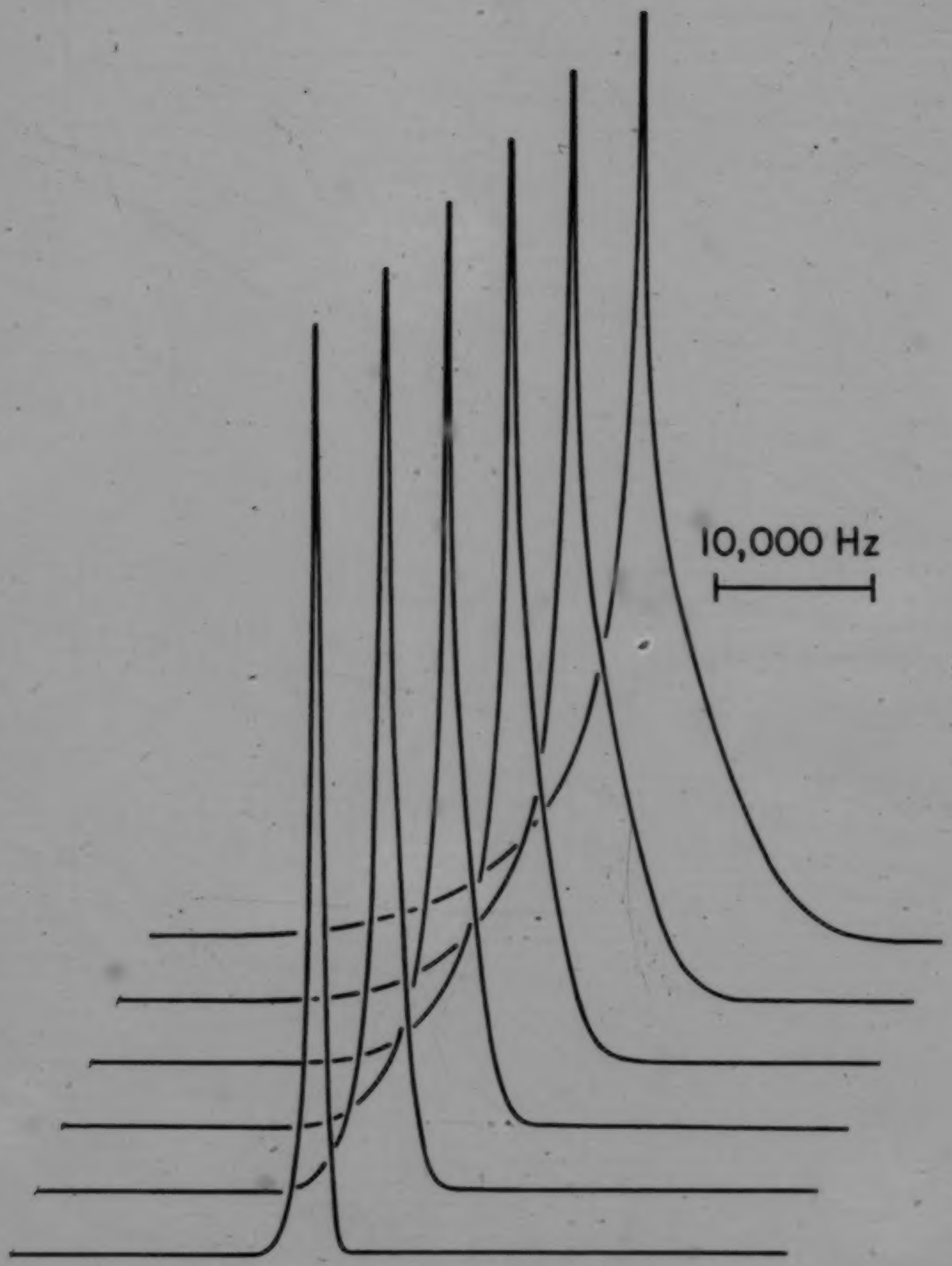


Fig. 13. The effect of increasing Sz on the theoretical line-shape where (from bottom to top) Sz=.05, .1, .15, .25, .3, and .4.

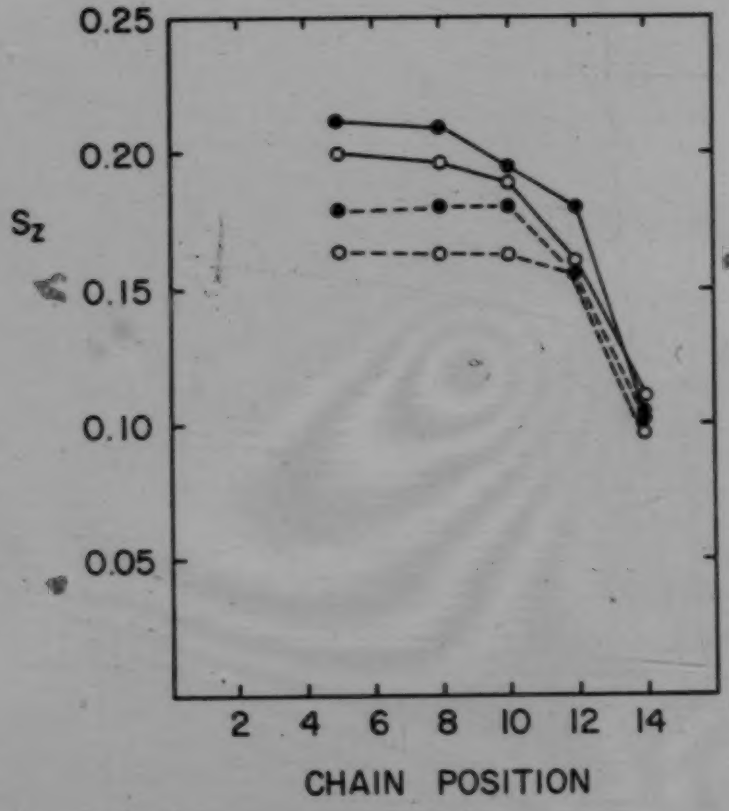


Fig. 14. The order profiles of straight-chain saturated and methyl branched fatty acid-enriched membranes at the growth temperature (37°C). Solid line ● (16:0), solid line ○ (15:0), broken line ● (16:0i), broken line ○ (16:0ai).

A second observation is that the order profile through the bilayer is also altered when a methyl branch is present (see also fig. 15). In this case, the presence of a methyl branch extends the plateau region of the order profile relative to that observed with the straight-chain fatty acids. The anteiso-branched fatty acid (16:0ai) extends this region to the 12-position of the chain at both 37° and 20°, while the iso-branched (16:0i) fatty acid markedly decreases the steepness of the slope of the order profile beyond the plateau region at 37°. It is important to note that the measured order parameters are those of the probe fatty acid, thus the presence of the methyl branch tends to increase the order of neighbouring fatty acids in the region of the methyl group relative to the other regions of the acyl chain, when compared to the case observed with the unbranched fatty acids. The same conclusions concerning the relative differences in the order profiles of these fatty acids may be made when all of the membrane lipids are in the liquid-crystalline state (see fig. 15). In these experiments the NMR spectra were collected at a temperature 15° above the midpoint of the membrane phase transition (Silvius and McElhaney, 1978a), which is assumed to represent a condition where the different membrane lipids are in the same physical state. Under these conditions the presence of a methyl branch again results in an increase in the length of the plateau region compared to the straight-chain fatty acid; however, there is a reversal in the observed average order under these conditions. The average order is now greatest in the methyl-branched fatty acids with 160ai>16:0i>16:0>15:0. This result suggests that the methyl-branched fatty acids, which are thought to have a disordering effect on the lipid bilayer compared to the straight-chain saturated fatty acids, are

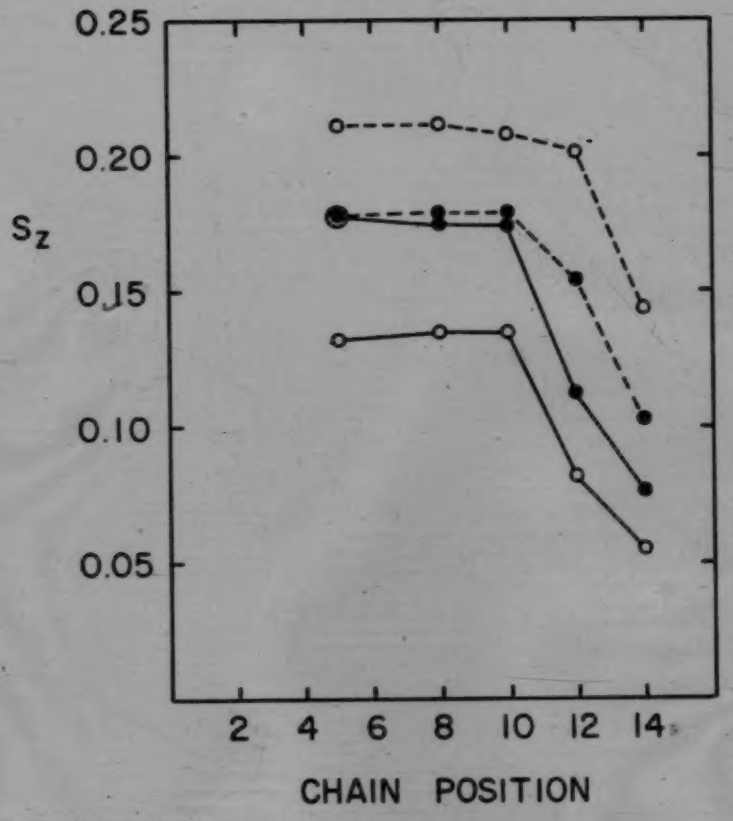


Fig. 15. The order profiles of straight-chain saturated and methyl branched fatty acid-enriched membranes at 15°C above the Tm of the membrane lipids. Solid line ● (16:0) at 45°C, solid line ○ (15:0) at 50°C, broken line ● (16:0i) at 37°C, and broken line ○ (16:0ai> at 20°C.

actually more ordered under similar thermodynamic conditions.

We have also looked at the effect of a trans-double bond on the acyl chain order in these membranes. At the growth temperature (see fig. 16), membranes enriched with fatty acids containing a trans-double bond (16:1tΔ^9, 18:1tΔ^9), are less ordered than membranes enriched with saturated fatty acids (15:0, 16:0). This is also consistent with the observed values of the phase transitions of the membranes, where the unsaturated fatty acid-enriched membranes have the lower Tm values (Silvius and McElhaney, 1978a). In contrast, the order observed in the 16:1tΔ^9-membranes is higher than the order seen for the 18:1tΔ^9-enriched membranes, which one would not expect using the criteria of the observed Tm values (Tm 16:1tΔ^9=6.7°, Tm 18:1tΔ^9=20.1°). The reason for this result is not clear, but is likely due to the difference in chain length between the probes and the 18:1tΔ^9, which does not exist in the case of the 16:1tΔ^9. This difference in chain length may result in "free spaces" in the region of the methyl termini of the shorter probe fatty acids which results in a decrease in the order of the probe molecules. Davis and co-workers (1980) have shown that the Tm's of mixed-acid (saturated-unsaturated) PC's are lower than predicted on the basis of the Tm's of the PC's composed of two (saturated or unsaturated) identical fatty acids. This in turn implies that the mixed acyl chain PC's would have less order than predicted from the Tm values of the non-mixed PC's. This question of the effect of probe-native acyl chain mismatch due to differences in chain length is not well understood, and to date very few observations concerning this problem have been reported in the literature.

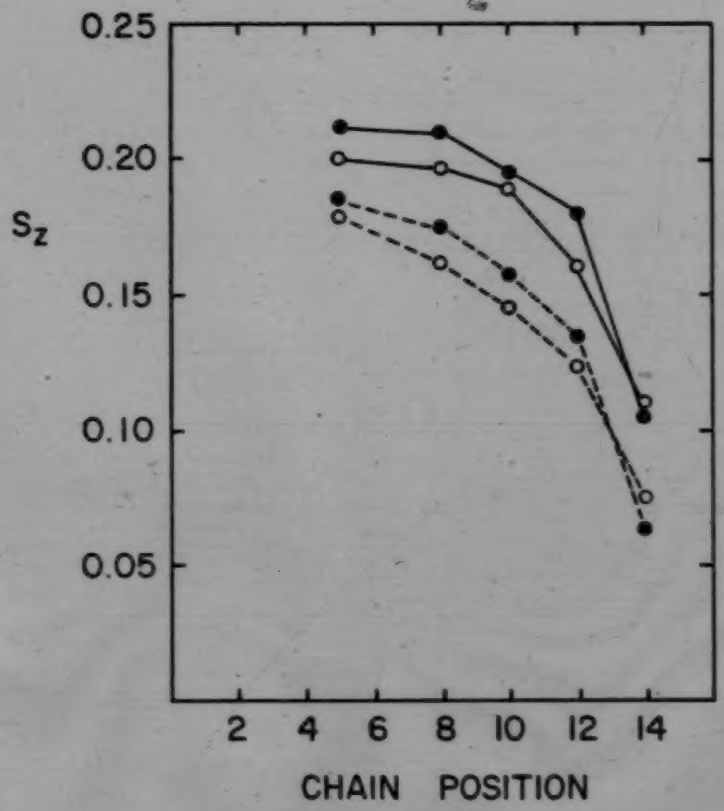


Fig. 16. The order profiles of straight-chain saturated and trans-unsaturated fatty acid-enriched membranes at the growth temperature (37°C). Solid line ● (16:0), solid line ○ (15:0), broken line ● (16:1tΔ^9), broken line ○ (18:1tΔ^9)

At the growth temperature the presence of the *trans*-double bond at the 9-position of the chain tends to decrease the length of the plateau region of the membrane bilayer (see fig. 16). This is much more apparent when one compares the order profiles of these unsaturated fatty acids to the order profiles of the saturated fatty acids when all of the membrane lipids are in the liquid-crystalline state (fig. 17). Thus where a methyl branch near the methyl terminus of a fatty acid increases the length of the plateau region, the presence of a *trans*-double bond decreases the length of the plateau region.

Under conditions where the membrane lipids are assumed to be in similar thermotropic (liquid-crystalline) states (15°C above the Tm), it is seen that the fatty acid 16:1tΔ^9 has a higher average order than either of the saturated fatty acids (16:0, 15:0), while the membranes enriched with 18:1tΔ^9 have an average order less than 16:0- but greater than 15:0-enriched membranes. These observations contrast with the studies of model membrane systems in which the effect of a *cis*-double bond on the order of the bilayer was observed. In this case, the *cis*-double bond increased the local order of the probe molecules, while our results indicate a *trans*-double bond decreases the local order. In addition, it was also found that the average order of the system was increased in the presence of either type of double bond compared to the order observed with a saturated fatty acid when the bilayers were in similar thermotropic conditions (Seelig and Seelig, 1977).

Figures 18 and 19 show the order profiles of these various membranes at temperatures below their phase transitions (15:0, 16:0, 18:1tΔ^9, 16:0i) or at 5°C for 16:1tΔ^9 and 16:0ai. For the latter two cases the phase transition begins at temperatures below 0°C. The

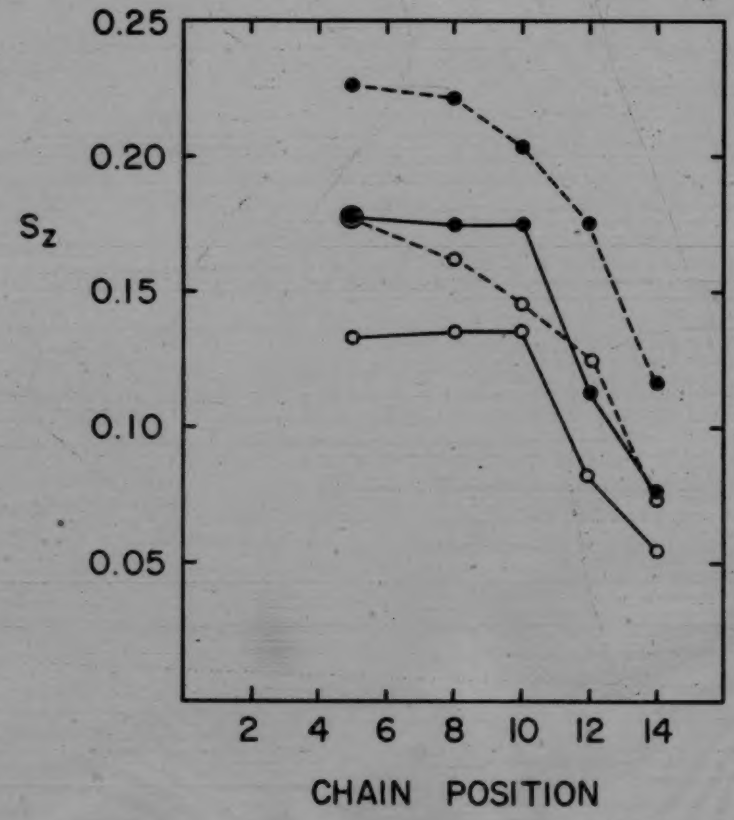


Fig. 17. The order profiles of straight-chain saturated and *trans*-unsaturated fatty acid-enriched membranes at 15°C above the Tm of the membrane lipids. Solid line ● (16:0) at 45°C, solid line ○ (15:0) at 50°C, broken line ● (16:1tΔ^9) at 20°C, and broken line ○ (18:1tΔ^9) at 34°C.

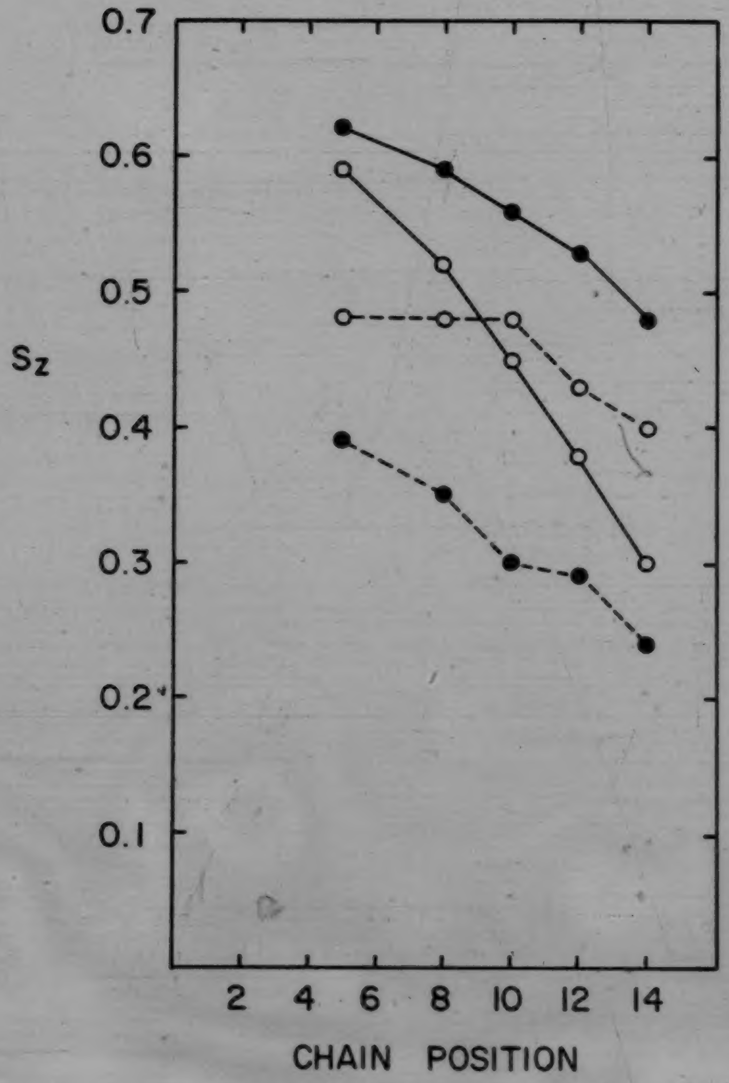


Fig. 18. The order profiles of straight-chain saturated and methyl branched fatty acid-enriched membranes at a temperature below the phase transition for 16:0, 15:0, and 16:0i, and at 5°C for 16:0ai. Solid line ● (16:0) at 20°C, solid line ○ (15:0) at 25°C, broken line ○ (16:0i) at 10°C, and broken line ● (16:0ai) at 5°C.

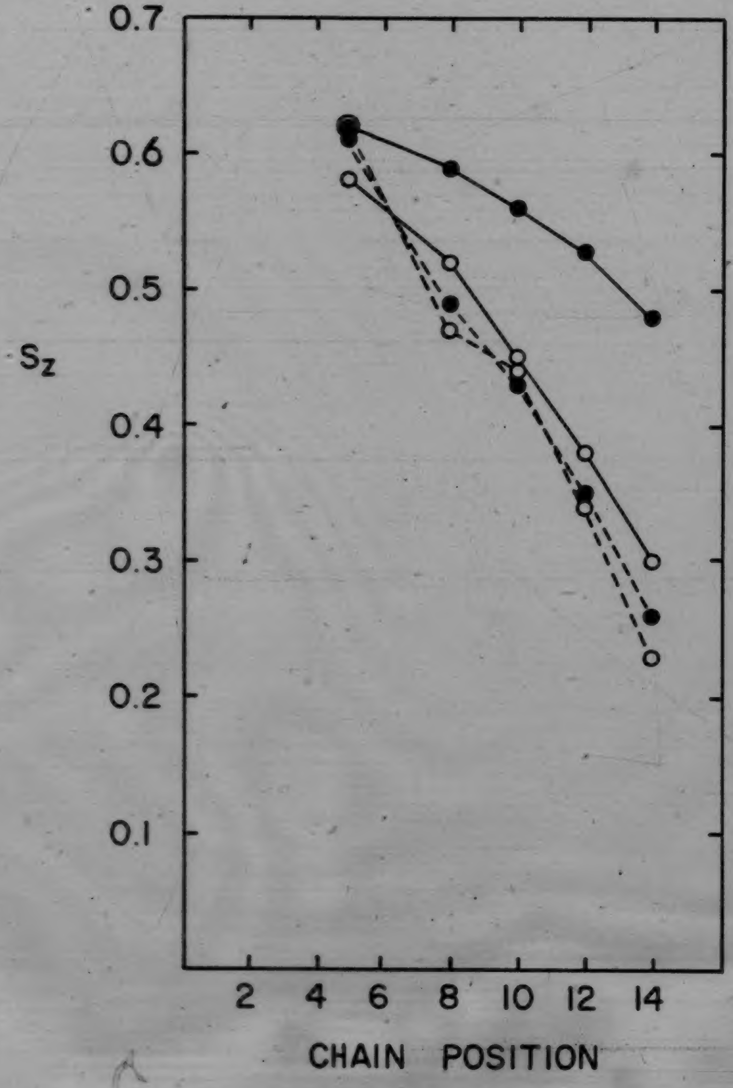


Fig. 19. The order profiles of straight-chain saturated and *trans*-unsaturated fatty acid-enriched membranes at a temperature below the phase transition for 16:0, 15:0, and 18:1tΔ^9, and at 5°C for 16:0ai. Solid line ● (16:0) at 20°C, solid line ○ (15:0) at 25°C, broken line ● (16:1tΔ^9) at 5°C and broken line ○ (18:1tΔ^9) at 10°C.

freezing point of D_2O is approximately 4°C, therefore the lowest temperature that spectra could be collected without freezing the sample was 5°.

In fig. 19 only 16:0ai-enriched membranes are not below their phase transition temperature. Under these conditions the straight-chain fatty acid-enriched membranes have the greatest average order; however, the plateau region is no longer present beyond the 5-position of the acyl chains, where the order profile indicates an increasingly larger decrease in the order beyond this position of the chain. For the 16:0i-enriched membranes in which all the lipids are in the gel state, the plateau region is still present. Thus, as was the case above with the phase transition, the methyl branch acts to maintain the plateau region and increases the order of the chains towards the methyl terminus relative to the other regions of the chain. In the case of the 16:0ai-enriched membranes, where the phase state of the lipids is mixed, the profile does not conform to what was previously observed. There is no plateau region beyond the 5-position of the chain, similar to the straight-chain fatty acid profiles; however, in the region of the methyl group there is a marked increase in the order relative to when this group is not present, which is consistent with the previously mentioned results.

The results obtained for the trans-unsaturated fatty acid-enriched membranes are presented in fig. 18. In this case the 18:1tΔ' membrane lipids are in the gel state while the 16:1tΔ' membrane lipids are in the region of their phase transition. The average order of the saturated fatty acids is greater than for either of the unsaturated fatty acids. In the region of the double bond there is an increase in the relative order, which is more apparent in the 18:1tΔ^9 profile. Thus the effect of the *trans*-double bond is to increase the steepness of the gradient of the order profile through the bilayer.

Figures 20 through 25 represent the order parameter profiles of membranes, enriched with different fatty acids mentioned above, at different temperatures; specifically above the phase transition, at the optimum growth temperature, and where possible below the phase transition, to directly compare the effect of temperature on the order profile of the individual fatty acid species. For the saturated fatty acids (figs. 20 and 21), the length of the plateau region tends to decrease as the temperature is decreased, with the plateau region the longest when the membrane lipids are in the liquid-crystalline state. As is the case with all the fatty acids studied, there is also a steady increase in the absolute order at each position of the chain as the temperature was decreased. This same trend is observed when a *trans*-double bond is present, in that the gradient of the order profile increases as the temperature was decreased; however, in this case a plateau region is not observed at any temperature beyond the 5-position of the chain (see figs. 24 and 25). This is opposite to what is observed in membranes enriched with methyl-branched fatty acids, where this group tends to maintain the presence of the plateau region, particularly in the case of the 16:0i-enriched membranes (see fig. 22). The results observed with the 16:0ai-enriched membranes (see fig. 23) indicate that in the region of the phase transition, where the membrane lipids are in a mixed phase state, the plateau region is not present. However, the results seen with the 16:0i-enriched membranes below the phase transition suggest that the plateau region would reappear in the 16:0ai membranes when all the

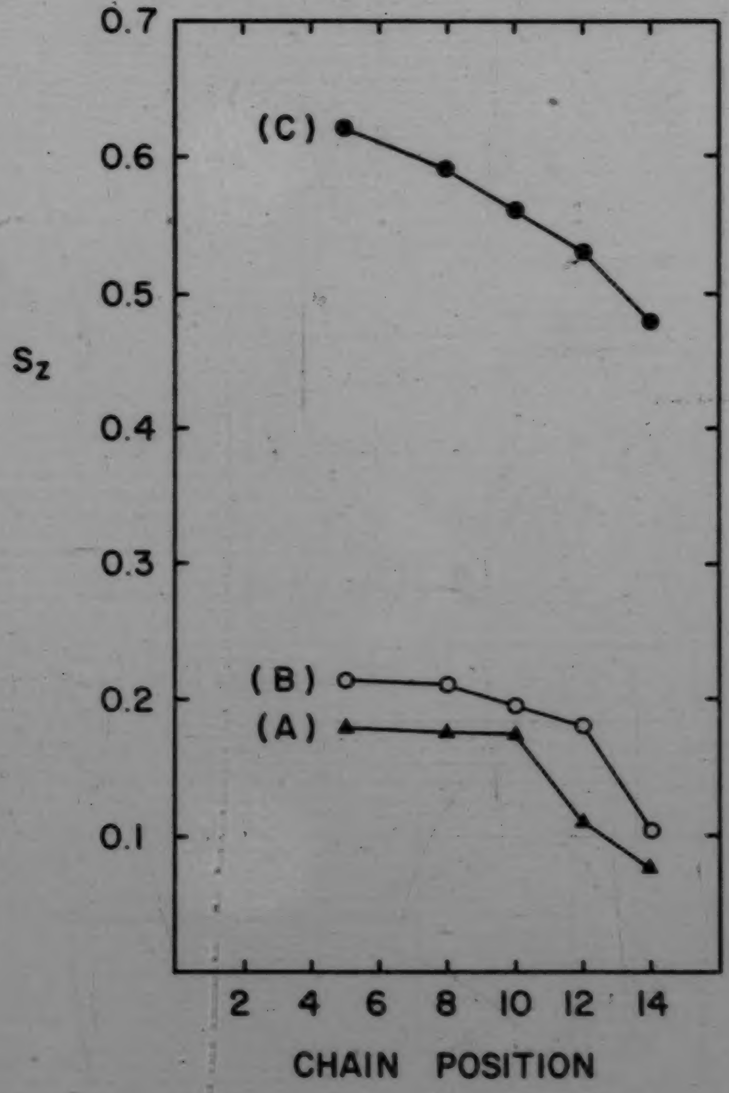


Fig. 20. The order profiles of 16:0-enriched membranes at various temperatures. (A) T=45°C, (B) T=37°C, and (C) T=20°C

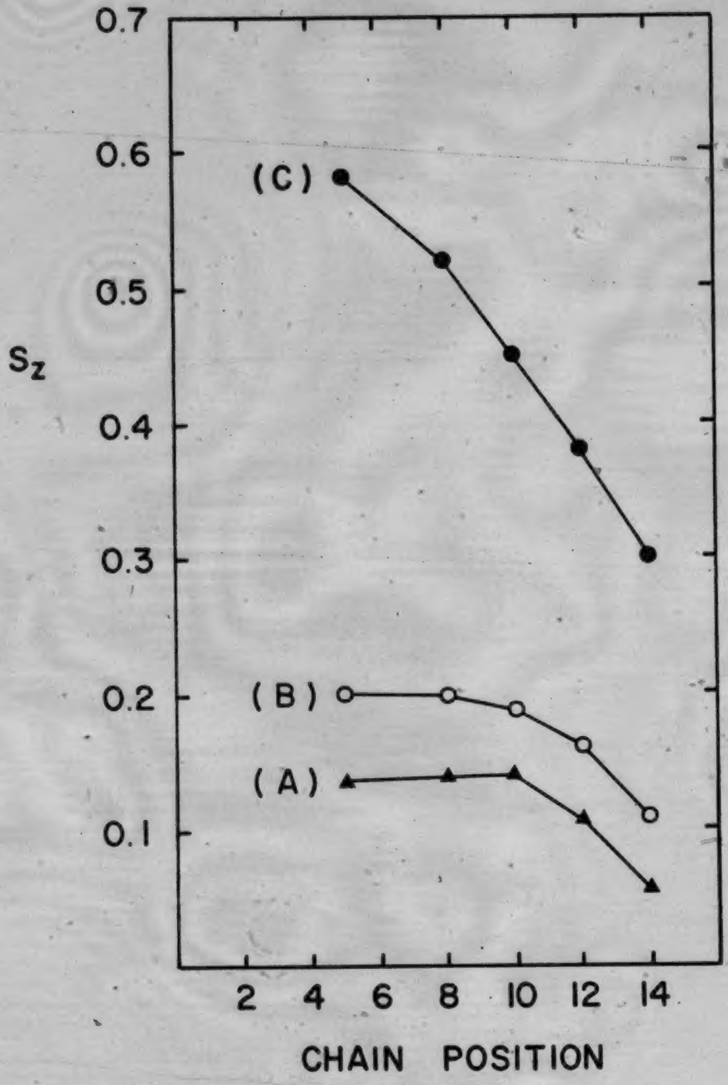


Fig. 21. The order profiles of 15:0-enriched membranes at various temperatures. (A) T=50°C, (B) T=37°C, and (C) T=25°C

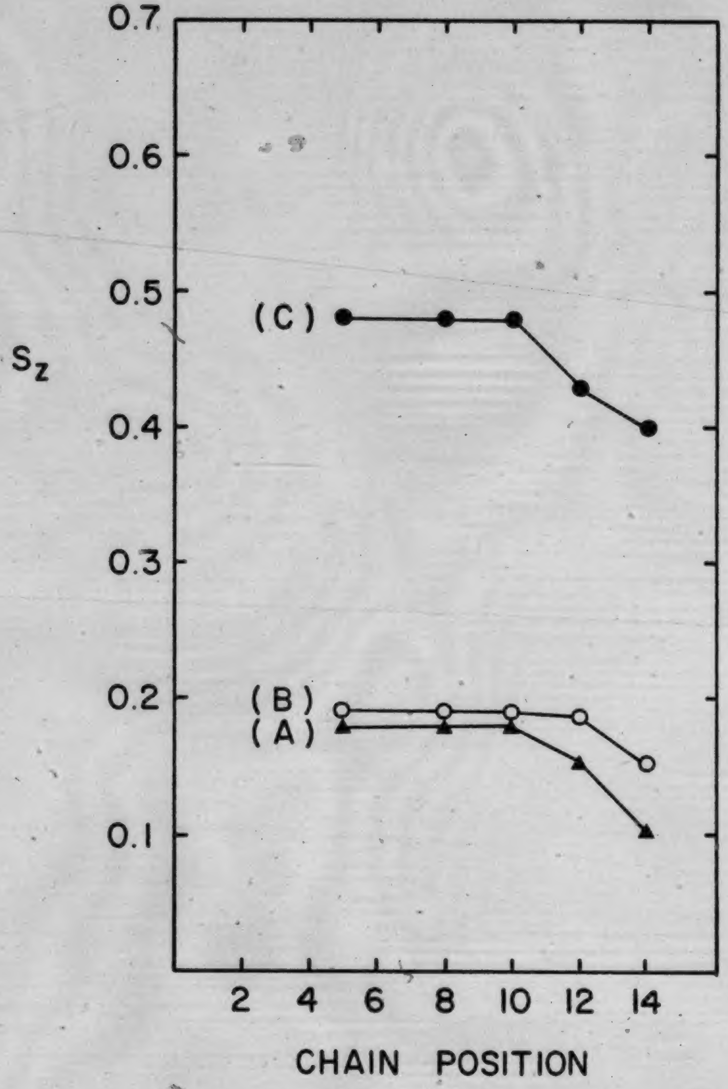


Fig. 22. The order profiles of 16:0i-enriched membranes at various temperatures. (A) T=37°C, (B) T=25°C, and (C) T=10°C.

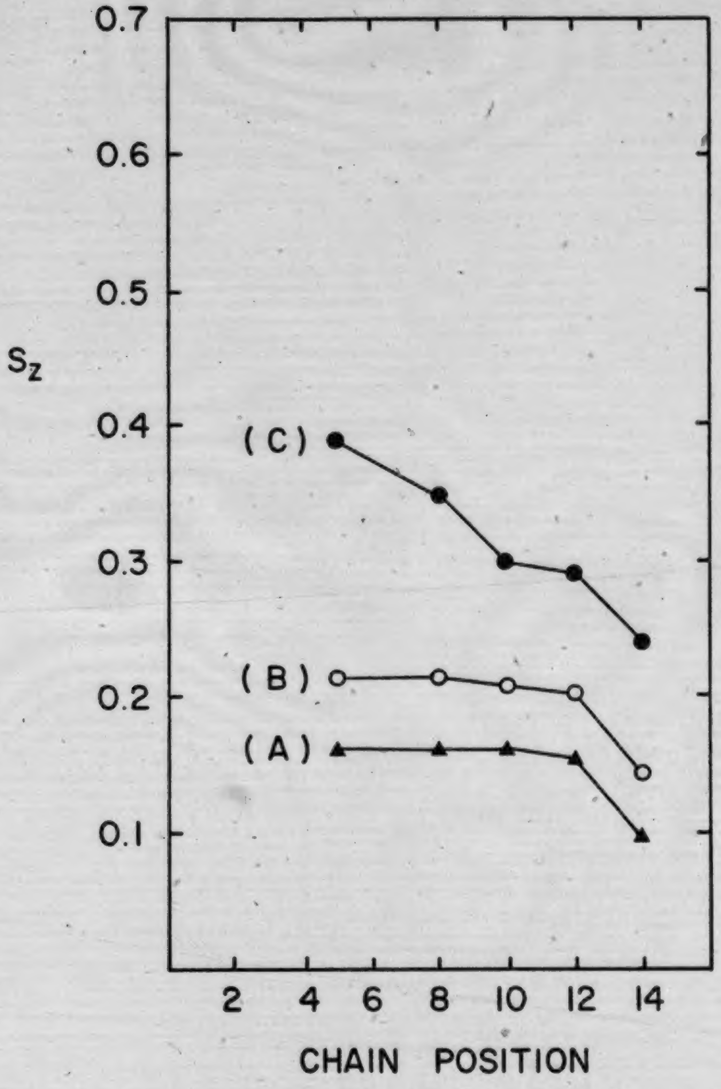


Fig. 23. The order profiles of 16:0ai–enriched membranes at various temperatures. (A) T=37°C, (B) T=20°C, and (C) T=5°C.

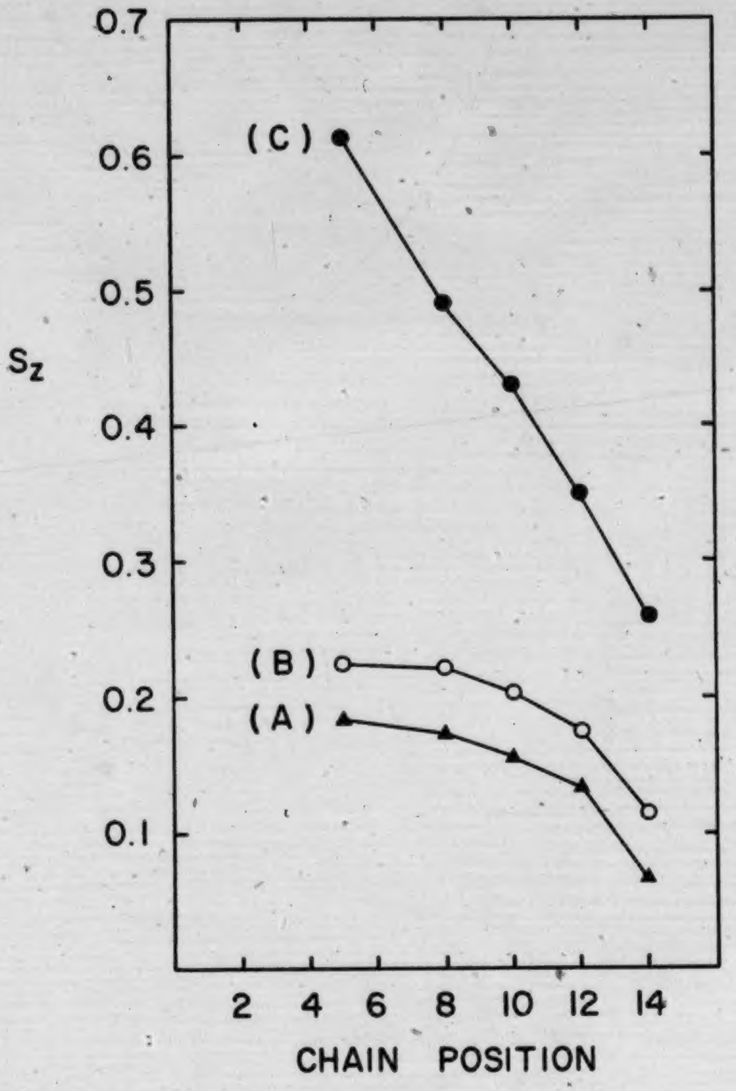


Fig. 24. The order profiles of 16:1t△'-enriched membranes at various temperatures. (A) T=37°C, (B) T=20°C, and (C) T=5°C.

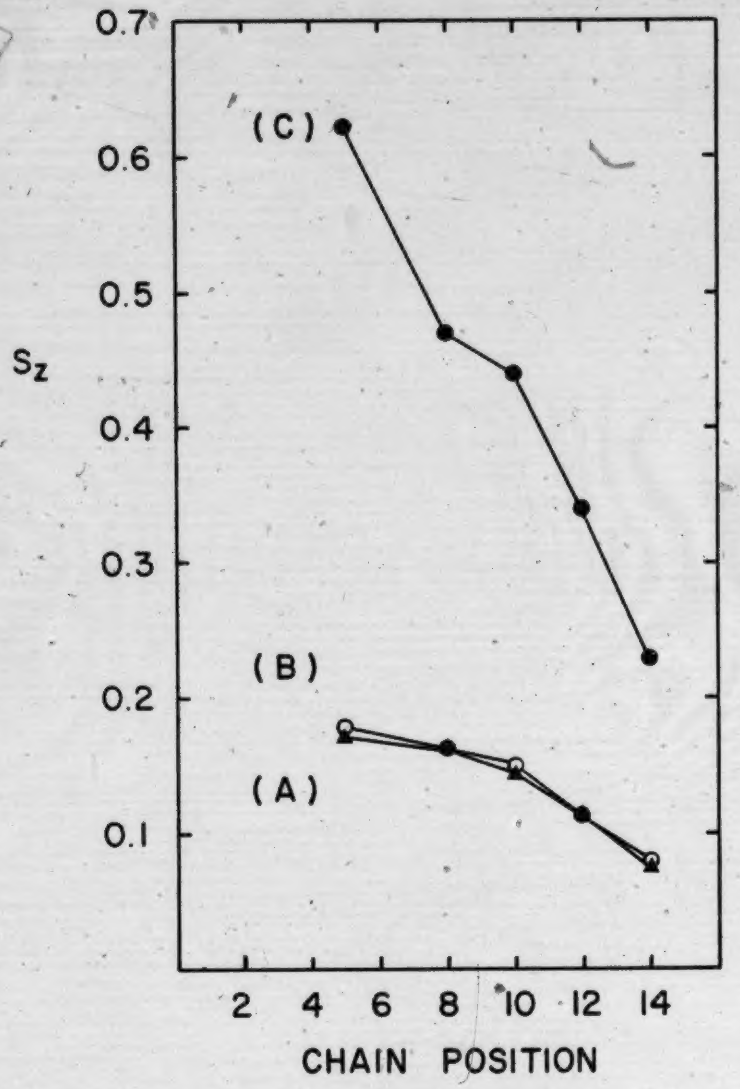


Fig. 25. The order profiles of 18:1tΔ'—enriched membranes at various temperatures. (A) T=37°C, (B) T=34°C, and (C) T=10°C.

lipids were in the gel state. This prediction could be tested by obtaining the order profile of the 16:0i membranes in the region of their phase transition, where one would predict that the plateau region would not be present according to the above hypothesis.

C. Conclusions

The results presented in this chapter attempt to address three questions raised in Chapter 1. The first question concerns the potential usefulness of A. laidlawii B as a biological system to study the effects of various fatty acid structures on the organization of the membrane lipids in the hydrocarbon core of the bilayer. This organism contains only one membrane, the plasma membrane, which is easily isolated from the rest of the cellular components (Silvius et al., 1977). In addition, a method for manipulating the fatty acid composition of this membrane has been determined such that the number of different lipid species present may be reduced from hundreds to approximately five, which vary only in the structure of their headgroups (Silvius et al., 1978). I have found that this organism will also incorporate monofluorinated fatty acids with little or not effect on the cells, and by combining these observations it is possible to obtain membranes consisting of almost entirely of one type of fatty acid together with a small amount of the fluorinated fatty acid to use as an NMR probe. The results to date suggest that, by using this system, a wide variety of fatty acids may be studied to determine their effects on the acyl chain organization in a biological system.

The second question involves the method used for the determination of the order parameter values from the observed spectra. A primary assumption is that the value of the parameter Δ_1 is independent of the temperature. Studies done by Niederberger and Seelig (1976) indicate that this number has a small temperature dependence when theoretical lineshapes are generated for ^{31}P-NMR spectra of phospholipid bilayers; however, the variation of this value over the temperature range studied is smaller than the error introduced into this value when determined using the procedure described in this chapter. Therefore, the value of Δ_1 is assumed to be constant in this study. An absolute error in either of the values $(\sigma_{\parallel}-\sigma_{\perp})$ or Δ_1 would result in a constant absolute error in the measured order parameter, in which all of the Sz values reported would be either overestimated or underestimated, but would not affect the relative values of Sz determined for the membrane samples. These are the only two fixed parameters that might be subject to this type of systematic error. The similarity of the order parameter values determined using ^{2}H-NMR and ^{19}F-NMR in the identical system (see Chapter 3) suggests that any error of this kind, if present, is relatively small.

The final point to consider is the effect the fatty acid structure has on the order of the membrane bilayer. From these studies it is clear that the structure of the fatty acid has a marked effect on the acyl chain organization in a bilayer. Although the fatty acids looked at in this study represent only a small number of the numerous different fatty acids found in biological membranes, some general conclusions concerning the effects of structure on the order may be made. If the straight-chain saturated fatty acids are used as a reference to compare the different types of fatty acids, then the addition of a methyl group near the methyl terminus of the chain tends to maintain and extend the plateau region above and likely below the phase transition of the membrane lipids, and as a consequence decrease the order gradient through the bilayer. Secondly, if we accept the assertion that at 15°C above the phase transition midpoint and at a temperature just below the onset of the phase transition the membrane lipids are in similar thermotropic states, then above the transition the methyl group increases the average order, while below the transition it decreases the average order of the acyl chains relative to the straight-chain fatty acids. In contrast, the introduction of a trans-double bond markedly decreases the plateau region of the chain, resulting in an increased gradient of order through the bilayer. These results, together with those found by Seelig and Seelig (1977), suggest that the cis- and trans-double bond have the opposite effect on the local ordering of neighbouring acyl chains but the same effect on the average order of the bilayer. They both increase the order under similar thermotropic conditions above the phase transition, but the trans-double bond acts to decrease the local ordering of the chains, whereas the cis-double bond acts to increase the local ordering. The effect of the cis-double bond on the order of gel state lipid has not been reported; however, our results with the trans-double bond indicate that, as was the case with the methyl group, the average order is now less than that observed in the straight-chain saturated enriched-membranes. All of these results suggest that an organism may not only control the fluidity of its membranes by varying the fatty acid structure of its membrane lipids (Saito and McElhaney, 1977), but also the magnitude of the acyl chain orientational order as well as the gradient of order, through the lipid bilayer.

V. BIBLIOGRAPHY

Ames,D.E. and Covell,A.N. (1963) J. Chem. Soc. (London) 775

Anderson, H.C. (1978) Ann. Rev. Biochem. 47, 359.

Bell, G.M., Mingins, J. and Taylor, J.A.G. (1978) Farady Transactions II, No. 1, 149.

Birdsall, N.J.M., Lee, A.G., Levine, Y.K. and Metcalfe, J.C. (1971) Biochim. Biophys. Acta. 241, 693.

Bladley, R.A., Martin, W.G. and Schneider, H. (1973) Biochemistry 12, 268.

Bligh, E.J. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911.

Bloom, M., Burnell, E.E., Valic, M.I. and Weeks, G. (1975) Chem. Phys. Lipids 14, 107.

Butler, K.W., Smith, I.C.P. and Schneider, H. (1970) Biochim. Biophys. Acta 363, 351.

Butler, K.W., Johnson, K.G. and Smith, I.C.P. (1978) Arch. Biochem. Biophys. 191, 289.

Cadenhead, D.A. and Muller-Landau, F. (1974) J. Colloid, Sci. 49, 131.

Cadenhead, D.A., Kellner, B.M.J. and Muller-Landau, F. (1975) Biochim. Biophys. Acta 382, 253.

Cameron, D.G., Casal, H.L., Gudgin, E.F. and Mantsch, H.H. (1980) Biochim. Biophys. Acta 596, 463.

Chen, R.F. (1967) J. Biol. Chem. 242, 1973.

Christie, W.W., Ed. (1973) Lipid Analysis, Pergamon Press, Oxford 137.

Cornell, B.A., Keniry, M., Hiller, R.G. and Smith, R. (1980) FEBS Lett. 115 , 134.

Davis, P.J., Coolbear, K.P. and Keough, K.M.W. (1980) Can. J. Biochem.

58, 851.

Davis, J.H. (1979) Biophys. J. 27, 336.

Davis, J.H., Nichol, C.P., Weeks, G. and Bloom, M. (1979) Biochemistry 18, 2103.

Dufourcq, J. and Lussan, C. (1972) FEBS Lett. 26, 35.

Engelman, D.M. (1971) J. Mol. Biol. 58, 153.

Fox, C.F. and Keith, A.D. (1972) Membrane Molecular Biology, Sinauer Associates, Stamford, Conn.

Gaber, B.P. and Peticolas, W.L. (1977) Biochim. Biophys. Acta 465, 260.

Gaber, B.P., Yager, P. and Peticolas, W.L. (1978) Biophys. J. 22, 191.

Gent, M.P.N., Armitage, I.M. and Prestegard, J.H. (1976) J. Amer. Chem. Soc. 98, 3749.

Gent, M.P.N. and Ho, C. (1978) Biochemistry 17, 2023.

Gent, M.P.N., Cottam, D.F. and Ho, C. (1978) Proc. Natl. Acad. Sci. 75, 630.

Guidotti, G. (1972) Arch. Intern. Med. 129, 194.

Hemminga, M.A. (1975) Chem. Phys. Lipids 14, 151.

Hubbell, W.L. and McConnell, H.M. (1971) J. Amer. Chem. Soc. 93, 314.

Janiak, M.J., Small, D.M. and Shipley, G.G. (1976) Biochemistry 15, 4575.

Jost, P.C., Nadakavukaren, K.K. and Griffith, O.H. (1977) Biochemistry 16, 3110.

Kang, S.Y., Gutowsky, H.S., Hsung, J.C., Jacobs, R., Tsoo, E.K., Rice, D. and Oldfield, E. (1979) Biochemistry 18, 3257.

Kohler, S.J. and Klein, M.P. (1977) Biochemistry 16, 519.

Lavialle, F. and Levin, I.W. (1980) Biochemistry 19, 6044.

Lentz, B.R., Baronholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521.

Lesshauer, W., Cain, J.E. and Blasie, J.K. (1972) Proc. Natl. Acad. Sci. 69 , 1499.

Levin, I.W. and Bush, S.F. (1981) Biochim. Biophys. Acta 640, 760.

Lichtenberg, O., Peterson, N.O., Girardet, J., Kainosko, M., Kroon, P.A., Seiter, C.H.A., Feigenson, G.W. and Chan, S.I.(1975) Biochim. Biophys. Acta 382, 10.

Lippert, J.L. and Peticolas, W.L. (1971) Proc. Natl. Acad. Sci. 68, 1572.

Longmuir, K.J., Capaldi, R.A. and Dalquist, F.W. (1977) Biochemistry 16, 5746.

Mabrey, S. and Sturtevant, J.M. (1978) Methods in Membrane Biology 9, 237.

McElhaney, R.N.M. and Tourtellette, M.E. (1970) Biochim. Biophys. Acta 202, 455.

McElhaney, R.N.M. (1974) J. Mol. Biol. 84, 145.

Mehring, M., Griffin, R.G. and Waugh, J.S. (1971) J. Chem. Phys. 55, 746.

Mendelson, R. and Maisano, J. (1978) Biochim. Biophys. Acta 506, 192.

Mendelson, R., Sunder, S. and Bernstein, H.J. (1976) Biochim. Biophys. Acta 443, 613.

Nichol, C.P., Davis, J.H., Weeks, G. and Bloom, M. (1980) Biochemistry 19, 451.

Niederberger, W. and Seelig, J. (1976) J. Amer. Chem. Soc. 98, 3704.

Oldfield, E., Keough, K.M. and Chapman, D. (1972) FEBS Lett. 20, 344.

Oldfield, E., Lee, R.W.R., Meadows, M., Dowd, S.R. and Ho, C. (1980) J. Biol. Chem. 255, 11652.

Overath, P., Brenner, M., Gulik-Krzywicki, T., Schechter, E. and Lettellier, L. (1975) Biochim. Biophys. Acta 389, 358.

Patel, K.M., Morrisett, S.D. and Sparrow, J.T. (1979) J. Lipid Res. 20, 674.

Peterson, N.O. and Chan, S.I. (1977) Biochemistry 16, 2657.

Podo, F. and Blasie, J.K. (1977) Proc. Natl. Acad. Sci. 74, 1032.

Pollack, J.D., Razin, S. and Cleverdon, R.C. (1965) J. Bacteriology 90, 617.

Rothfield, L.I. and Romeo, D. (1971) Structure and Function of Biology Membranes, p25.

Saito, Y. and McElhaney, R.N.M. (1977) J. Bacteriology 132, 485.

Schreier, S., Polnaszek, C.F. AND Smith, I.C.P. (1978) Biochim. Biophys. Acta 515, 375.

Seelig, J. and Niederberger, W. (1974) Biochemistry 13, 1585.

Seelig, A. and Seelig, J. (1974) Biochemistry 13, 4839.

Seelig, A. and Seelig, J. (1977) Biochemistry 16, 45.

Seiter, C.H. and Chan, S.I. (1973) J. Amer. Chem. Soc. 95, 7541.

Shinitzky, M., Dianoux, A.C., Gitler, C. and Weber, G. (1971) Biochemistry 10, 2106.

Shinitzky, M. and Baronholz, Y. (1978) Biochim. Biophys. Acta 515, 367.

Silvius, J.R. and McElhaney, R.N.M. (1978a) Can. J. Biochem. 56, 462.

Silvius, J.R., Mak, N. and McElhaney, R.N.M. (1978) Biochim. Biophys. Acta 597 , 199.

Silvius, J.R. and McElhaney, R.N.M. (1978b) Nature 272, 645.

Silvius, J.R. Jinks, D.C. and McElhaney, R.N.M. (1977) In: Microbiology (Schlessinger, D., Ed.) p. 10, Amer. Soc. for Microbiol., Washington, D.C.

Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720.

Singer, S.J. (1971) Structure and Function of Biological Membranes, Acad. Press, New York p145.

Smith, I.C.P. (1979) Can. J. Biochem. 57, 1.

Stankowski, S. and Gruenwald, B. (1980) Biophys. Chem. 12, 167.

Stockton, G.W., Johnson, K.G., Butler, K.W., Polnaszek, C.F., Cyr, R Smith, I.C.P. (1975) Biochim. Biophys. Acta 401, 535.

Stockton, G.W., Johnson, K.G., Butler, K.W., Tulloch, A.P., Boulanger, Y., Smith, I.C.P., Davis, J.H. and Bloom, M. (1977) Nature 269, 267.

Sturtevant, J.M., Ho, C. and Reiman, A. (1979) Proc. Natl. Acad. Sci. 76, 2239.

Taylor, M.G. and Smith, I.C.P. (1980) Biochim. Biophys. Acta 599, 140.

Terry, M.G., Engelman, D.M. and Morowitz, H.I. (1967) Biochim. Biophys. Acta 135, 391.

Thulborn, K.R. and Sawyer, W.H. (1978) Biochim. Biophys. Acta 511, 125.

Tilley, L., Thulborn, K.R. and Sawyer, W.H. (1979) J. Biol. Chem. 254, 2592.

Trauble, H. (1971) J. Membr. Biol. 4, 193.

Ulmius, J., Wennerstrom, H., Lindblom, G. and Arvidson, G. (1975) Biochim. Biophys Acta 389, 197.

Vogel, A.I., (1956) A Text Book of Practical Organic Chemistry, Longmans, Green and Co. Ltd., London, pp163-173.

VI. APPENDIX 1

The computer program given below is written in basic, and was used to generate the theoretical lineshapes to fit the experimental spectra and determine the order parameter values, Sz. This program, written by Dr. Brian Sykes, follows the theory described in chapter 2.

```
100 dim s(513),v(101),p(101),d(513),q(2048),p8$(3),k7$(3)
110 dim f(513)
120 dim e(201)
130 let d3=-.00008
140 let c=2000
150 let 17=2
160 print "write the desired ntcftb file-xxx .xxx"
170 input k7$(0),k7$(1)
180 let 17=17+1
190 call bdefine (17,k7$)
200 print "what is the field strength in hertz"
210 input vo
220 print "what is the sweep-width (+/-)"
230 input t
240 print "what is the value of d0"
250 input d0
260 print "what is the value of dl"
270 input dl
280 print "what is the value of sl"
290 input sl
```

```
300 let a=0

310 call fread(17,q,352)

320 call fread(17,q,2048)

330 let n9=2048

340 call faint (n9,1)

360 let h1=48.828

370 let h2=(50000-t)/h1

380 let h3=2*t/200

390 let h4=h3/h1

400 for y=1 to 201

410 let h5=h2+(y-1)*h4

420 let q(y)=q(h5)

430 next y

440 call fknob(k2,1)

450 call fknob (18,2)

460 let v1=d3*vo*s1

470 let c1=1.5*abs(v1)

480 for j=1 to 100

490 let v(j)=-1*v1-(j-1)*c1/100

500 let p(j)=1/sqr(1-2*v(j)/v1)

510 let d(j)=d0+abs(d1*s1*v(j)/v1)

520 next j

530 for i=1 to 201

540 let s(i)=0

550 let f(i)=t-(i-1)*t/100

560 for k=1 to 100

570 let e1=((f(i)-v(k))*(f(i)-v(k))/(2*d(k)*d(k))
580 if el>20 then 620

590 let el=-1*el

600 let s3=el+log (p(k)/d(k))

610 let s(i)=s(i)+exp (s3)

620 next k

630 print s(i),

640 next i

650 for i=0 to 200

660 let d(i)=s(i)*k2*c

670 next i

680 for y=1 to201-a

690 let q(y)=q(y+a)

700 next y

710 for y=201-a to 201

720 let q(y)=0

730 next y

740 go to 840

750 for y=a+1 to 201

760 let e(y)=q(y-a)

770 next y

780 for y=1 to a

790 let e(y)=0

800 next y

810 for y=1 to 201

820 let q(y)=e(y)

830 next y

840 call fdisp (q,201,3)
```

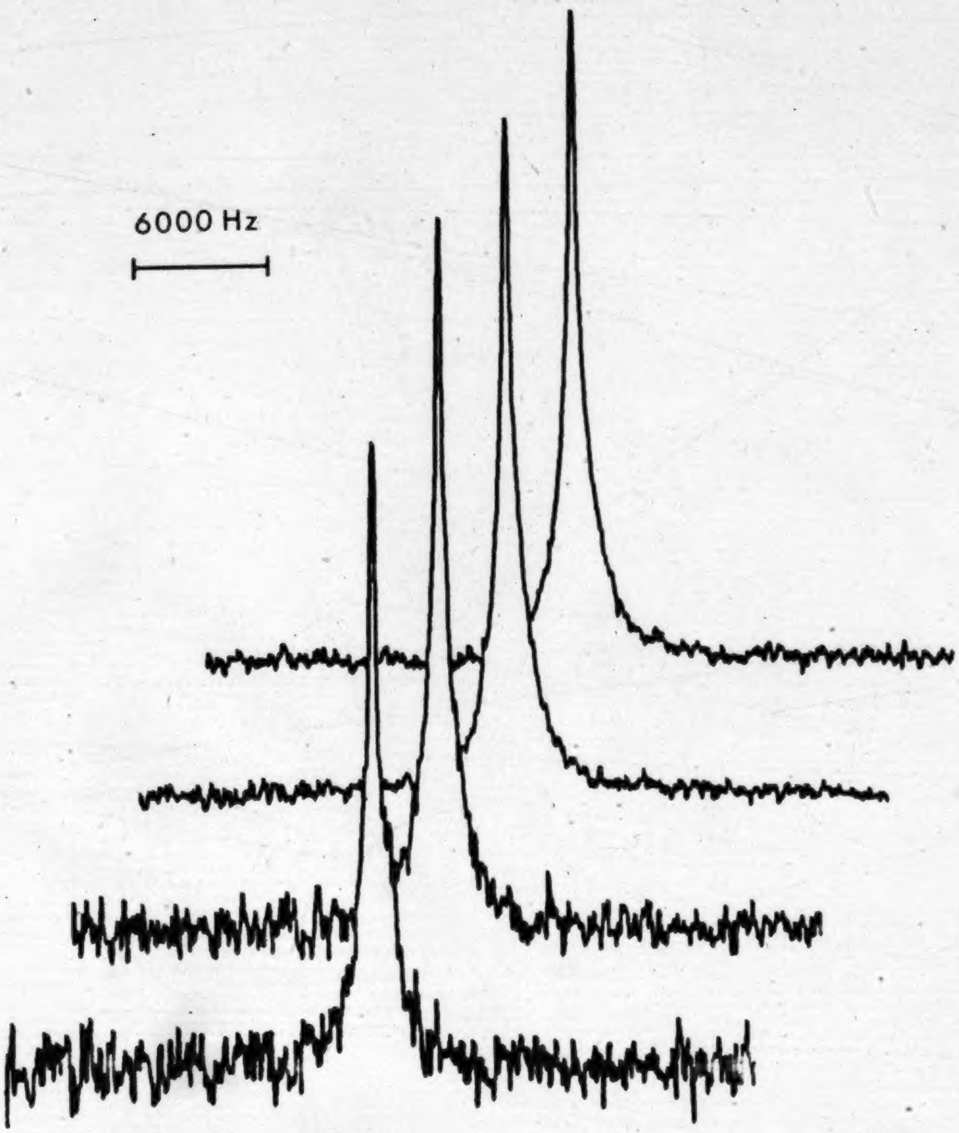
```
850 call fknob (s9,1)

860 if abs(s9-k2)>2 then 910

870 call fdisp (d,201,3)

880 call fknob (l9,2)

890 if abs (l9-l8)>2 then 960

900 go to 840

910 for i=0 to 511

920 let d(i)=s(i)*s9*c

930 next i

940 let k2=s9

950 go to 840

960 input u$

970 call fknob (l9,2)

980 let l8=l9

990 if u$="sr" then 1110

1000 if u$ ="c" then 840

1010 if u$="z" then 1280

1020 if u$="t" then 1600

1030 if u$="cp" then 1170

1040 if u$="sf" then 1140

1050 if u$="pf" then 1260

1060 if u$="na" then 160

1070 if u$="sl" then 1080

1080 print "what is the no. of points to shift spectrum left"

1090 input a

1100 go to 680

1110 print "what is the no. of points to shift spectrum right"
```

```
1120 input a
1130 go to 750
1140 print "what is the new scaling factor"
1150 input c
1160 go to 650
1170 print "the previous values of d0,d1,s1,were";d0,d1,s1
1180 print "indicate the desired new value of d0"
1190 print "beside the first ? (return) the new value of d1"
1200 print "beside the second ? and the new value of s1"
1210 print "beside the third ?"
1220 input d0
1230 input d1
1240 input s1
1250 go to 460 1260 print "the values of d0,d1 s1 are
";d0,d1,s1
1260 print "the values of d0,d1,s1
are";d0,d1,s1;"respectively"
1270 go to 840
1280 for y=-1*t to t step t/100
1290 let s(201-100*(y+t)/t-.8)=y
1300 next y
1310 let p8$(0)="hz"
1320 call fscale (q,6,201,1)
1330 call fscale (d,6,201,1)
1340 let s(201)=t
1350 let d(202)=q(202)
1360 print "how maney inches would you like the x-axis to be"
```

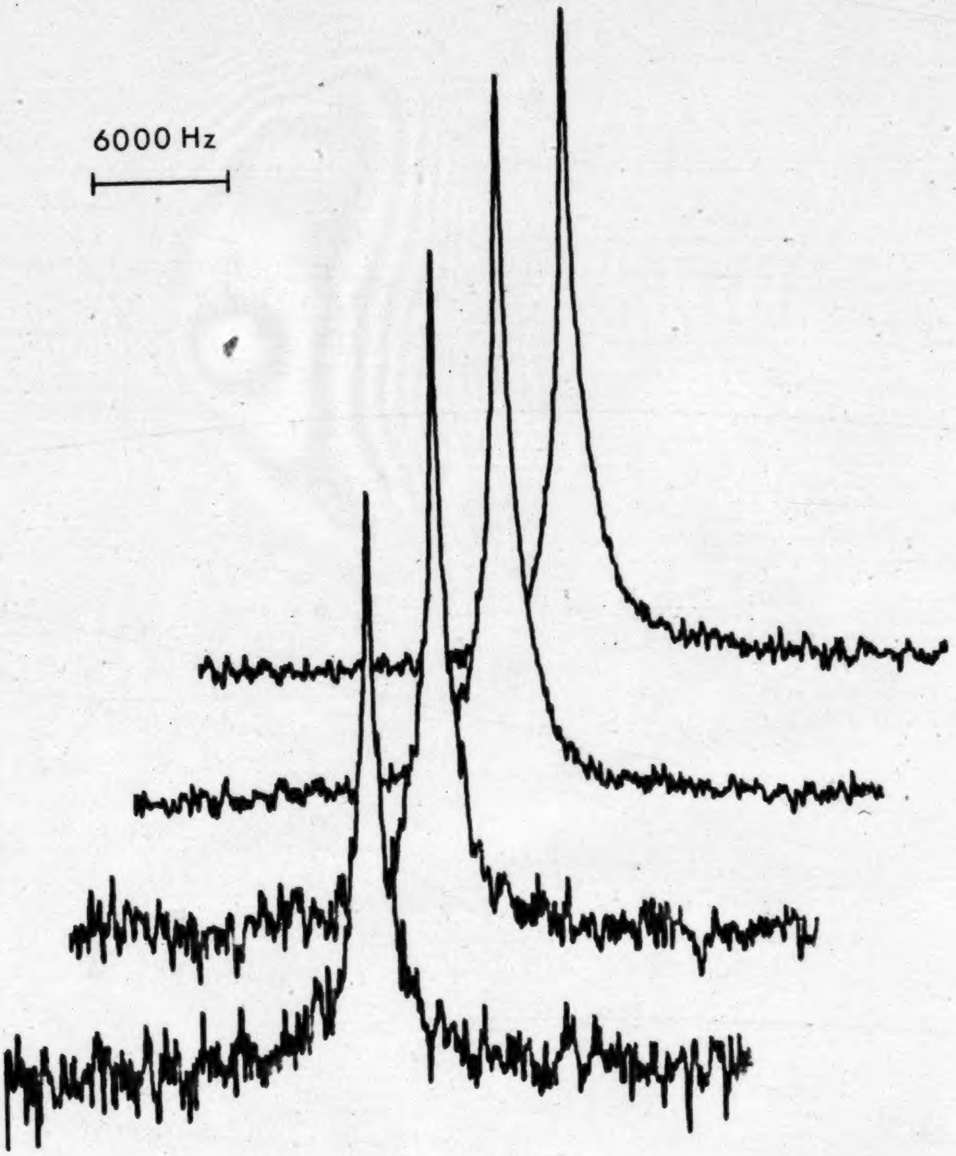
```
1370 input z1
1380 let s(202)=-2*t/z1
1390 call plots
1400 call faxis (0,1,p8$,2,z1,0,t,-2*t/z1,5,3)
1410 print "do you want only theoretical curve plotted"
1411 print "yes (1) or no (no)"
1420 input z2
1430 if z2=1 go to 1460
1440 call fplot (0,3,5,-3)
1450 call fline (s,q,201,1,0,0)
1460 call fdplot (0,2,-3)
1470 call fline (s,d,201,1,0,0)
1480 let c4$="d0="
1490 let c2$="d1="
1500 let c3$="s1="
1510 call fsymbol (0,8,.125,c4$,0,3)
1520 call fnumber (.25,8,.125,d0,0,8,4)
1530 call fsymbol (0,7.75,.125,c2$,0,3)
1540 call fnumber (.25,7.75,.125,d1,0,10,8)
1550 call fsymbol (0,7.50,.125,c3$,0,3)
1560 call fnumber (.25,7.50,.125,s1,0,8,4)
1570 print "do you want to do a new analysis (1) or "
1580 print "terminate (2)"
1590 input b
1600 if b=1 then 160
1610 end
```

VII. APPENDIX 2

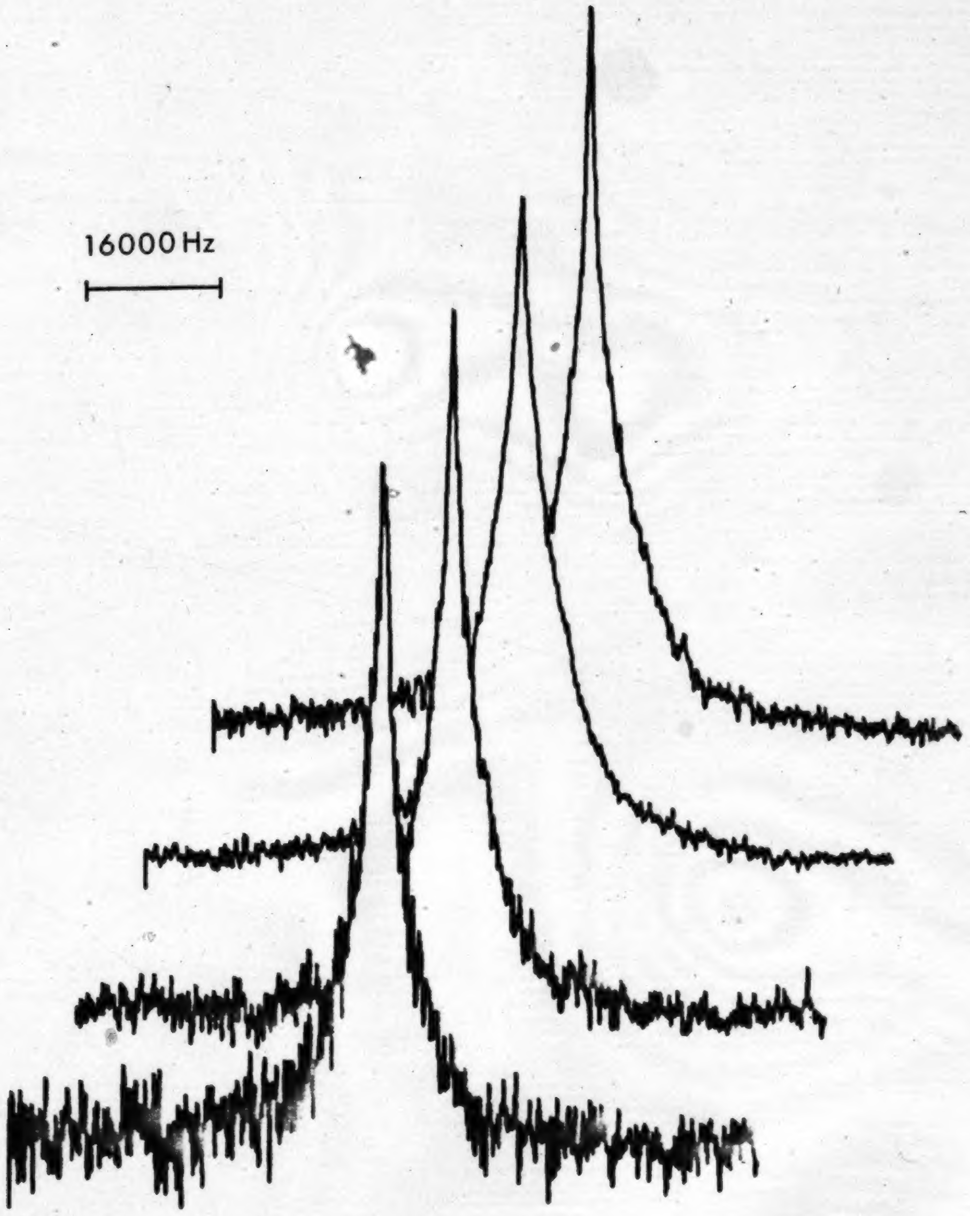
This appendix gives the NMR spectra collected for the membrane samples discussed in this work . The first six pages contain the spectra of membranes enriched with 16:0 in the absence of avidin supplemented with increasing amounts of either 5or 14-fluoropalmitic acid. The remaining spectra are of the membrane samples enriched with the various fatty acids looked at in this study, and whose order parameter profiles are given in chapter four.



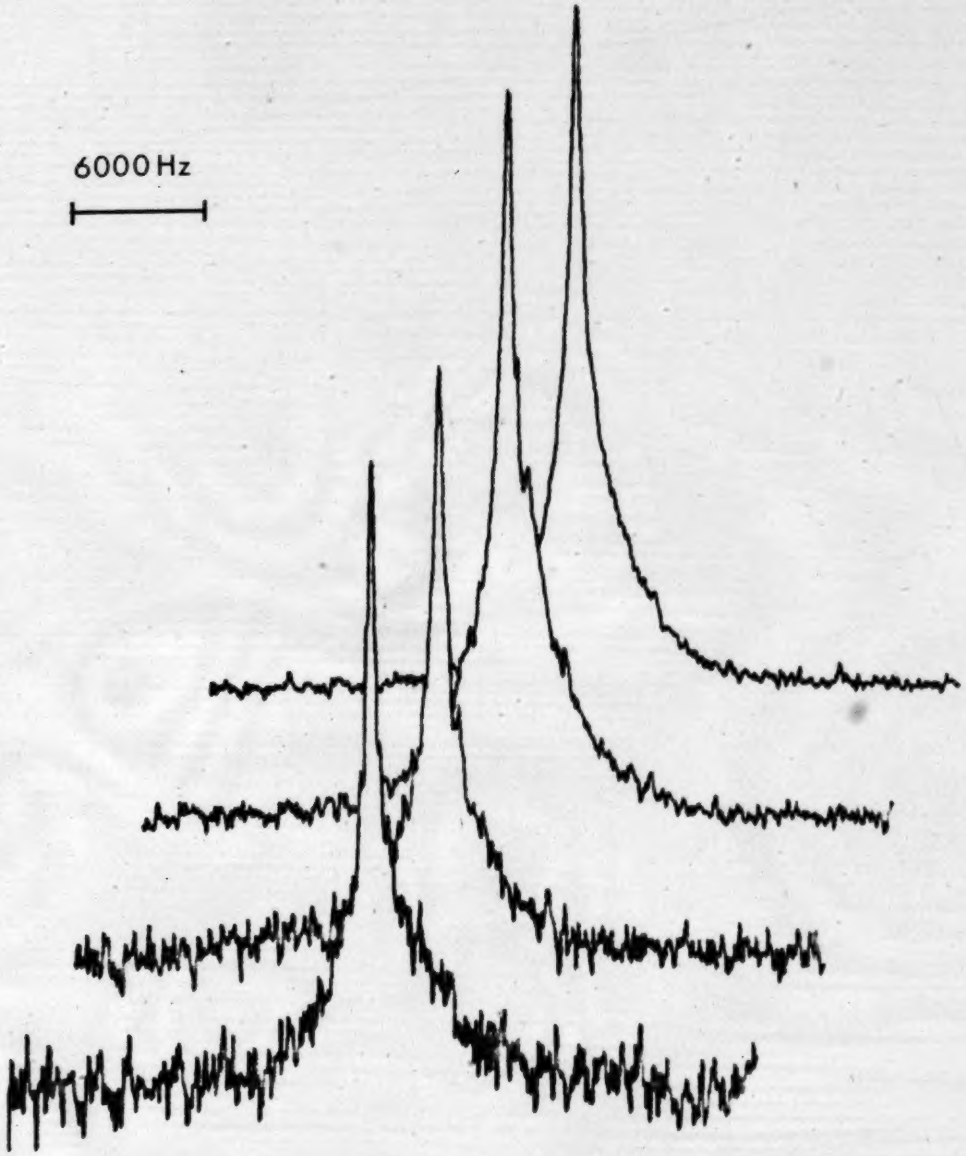
16:0-enriched membranes from cells grown in the absence of avidin containing approximately (from bottom to top) 5,15,25, and 50 mole % of 14-monofluoropalmitic acid at 45°C



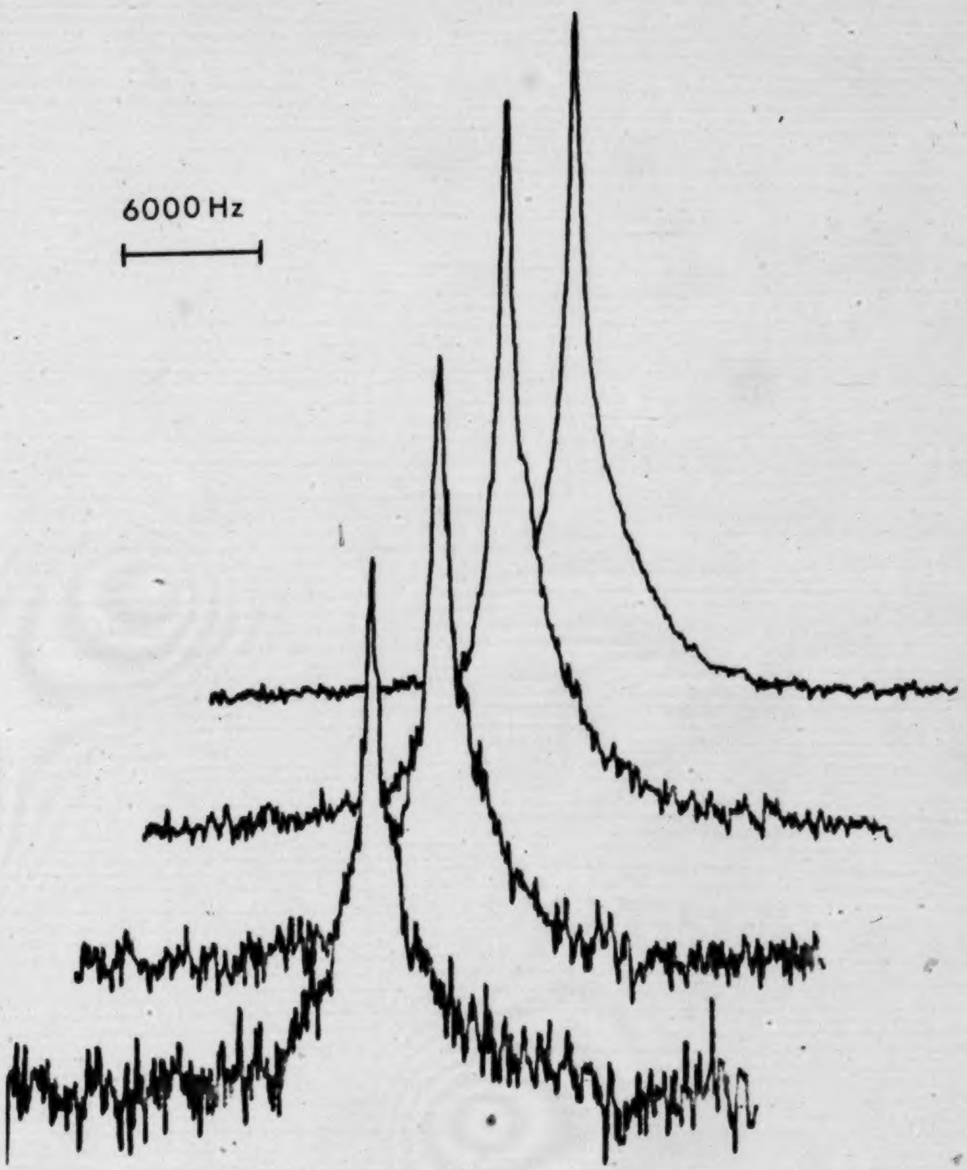
16:0-enriched membranes from cells grown in the absence of avidin containing approximately (from bottom to top) 5,15,25, and 50 mole % 14-monofluoropalmitic acid at 37°C



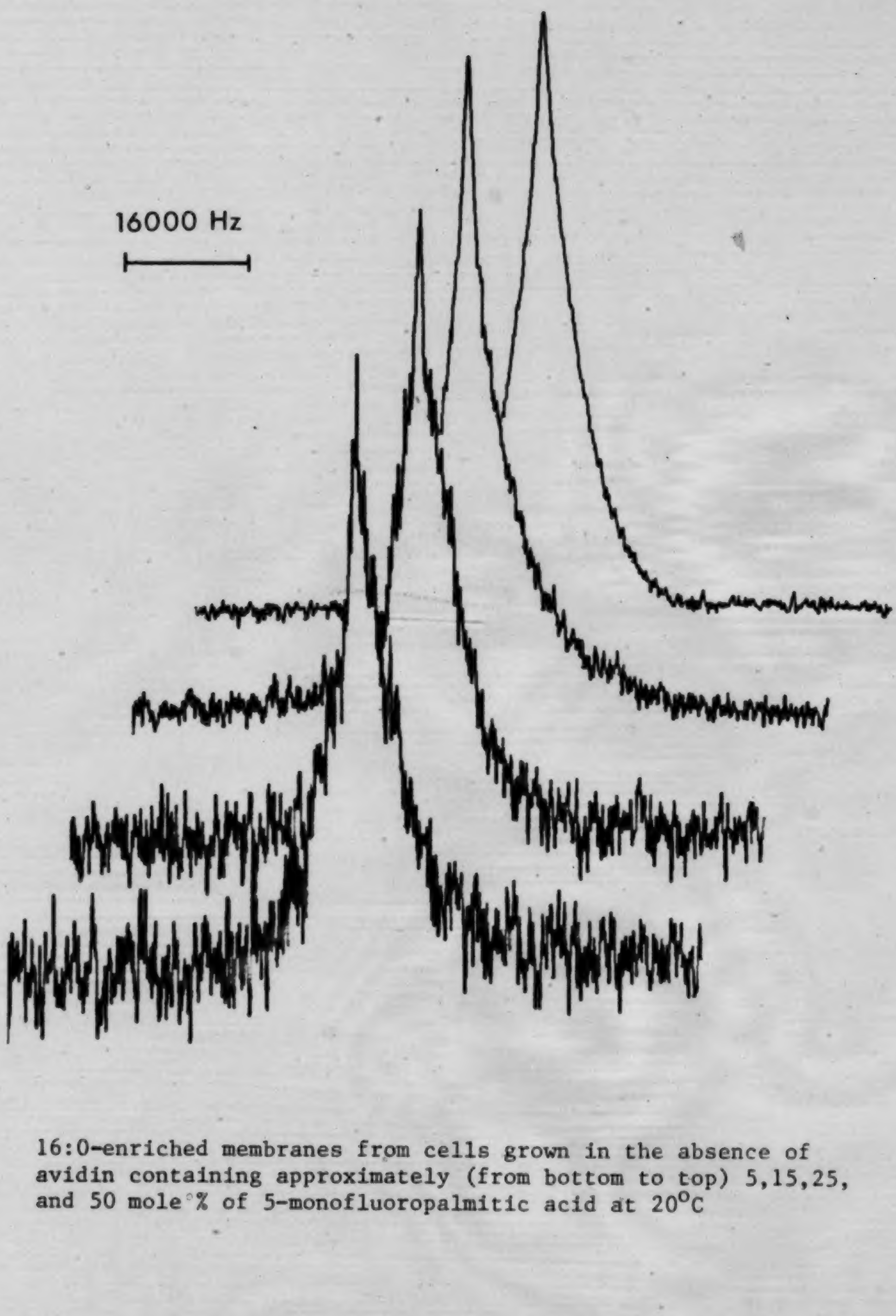
16:0-enriched membranes from cells grown in the absence of avidin containing approximately (from bottom to top) 5,15,25, and 50 mole % 14-monofluoropalmitic acid at 20°C



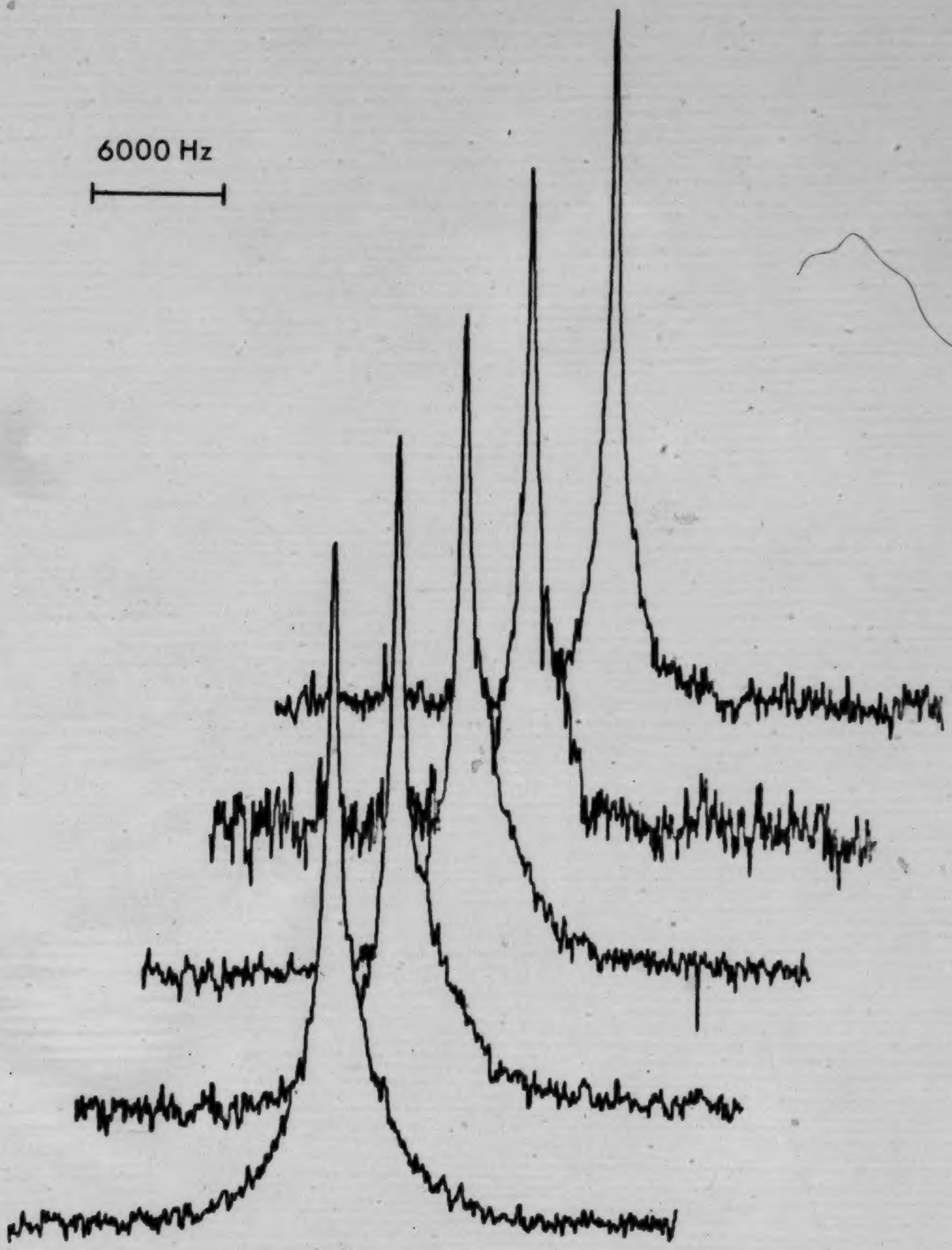
16:0-enriched membranes from cells grown in the absence of avidin containing approximately (from bottom to top) 5,15,25, and 50 mole % of 5-monofluoropalmitic acid at 45°C



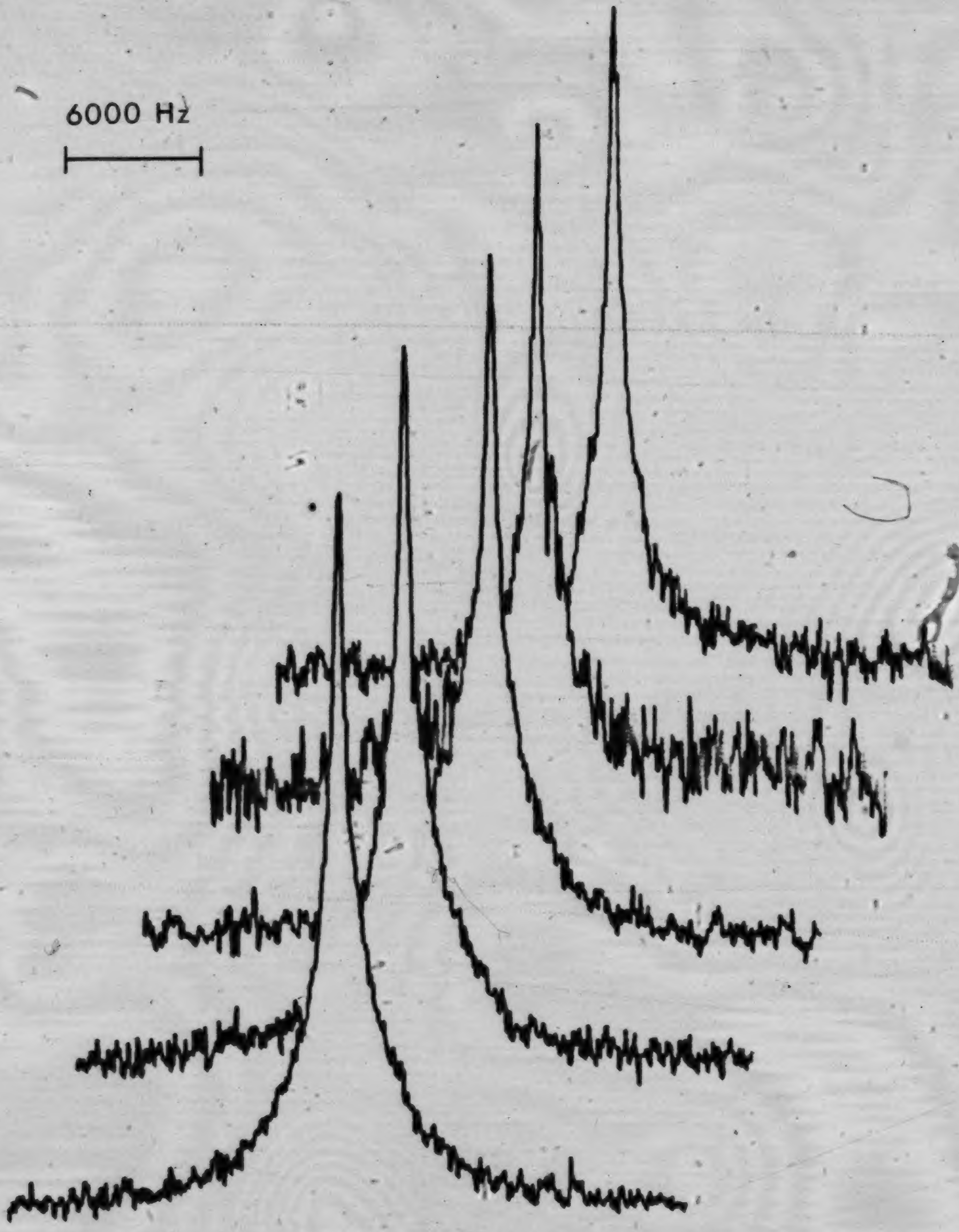
16:0-enriched membranes from cells grown in the absence of avidin containing approximately (from bottom to top) 5,15,25, and 50 mole % of 5-monofluoropalmitic acid at 37°C



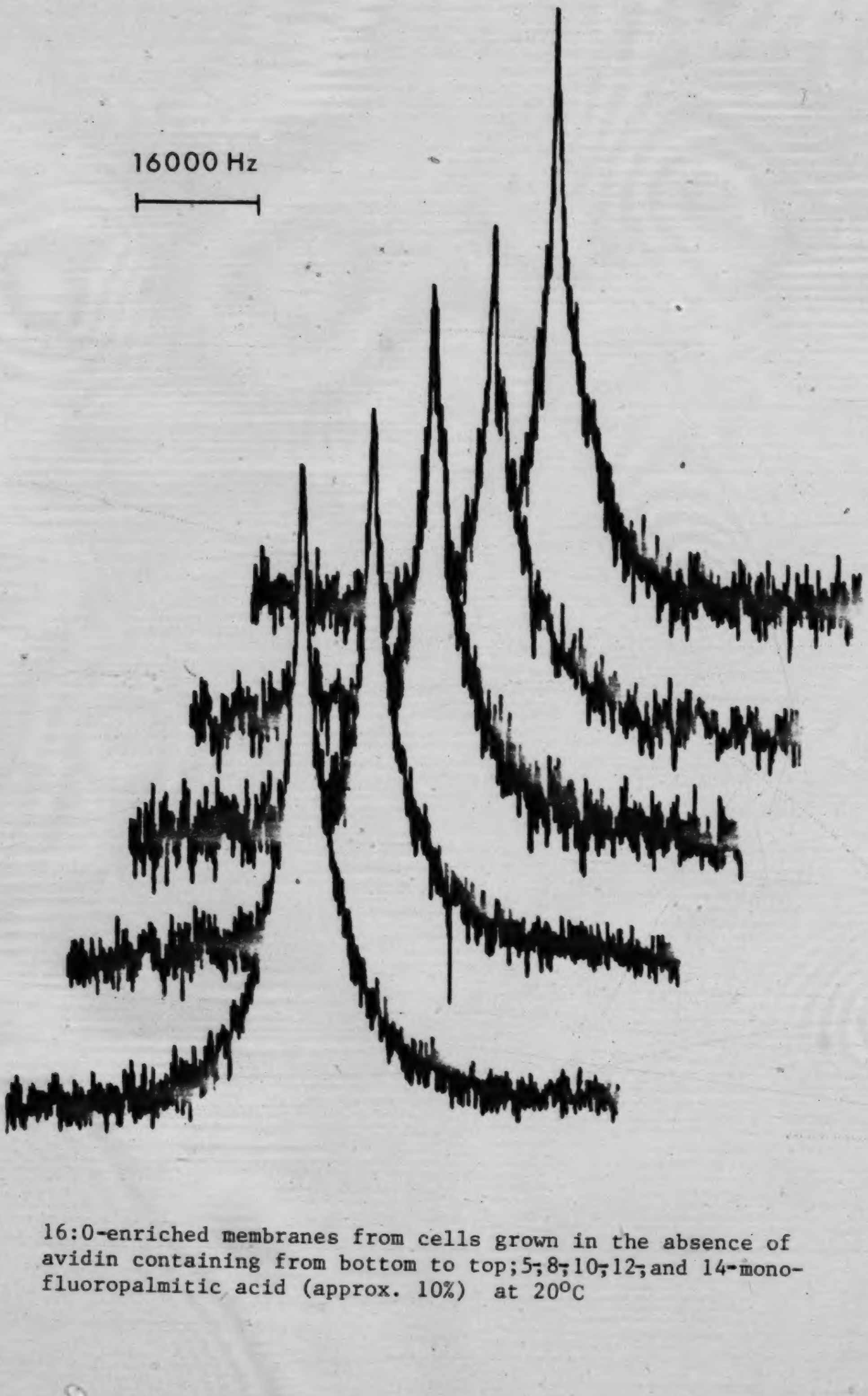
16:0-enriched membranes from cells grown in the absence of avidin containing approximately (from bottom to top) 5,15,25, and 50 mole % of 5-monofluoropalmitic acid at 20°C



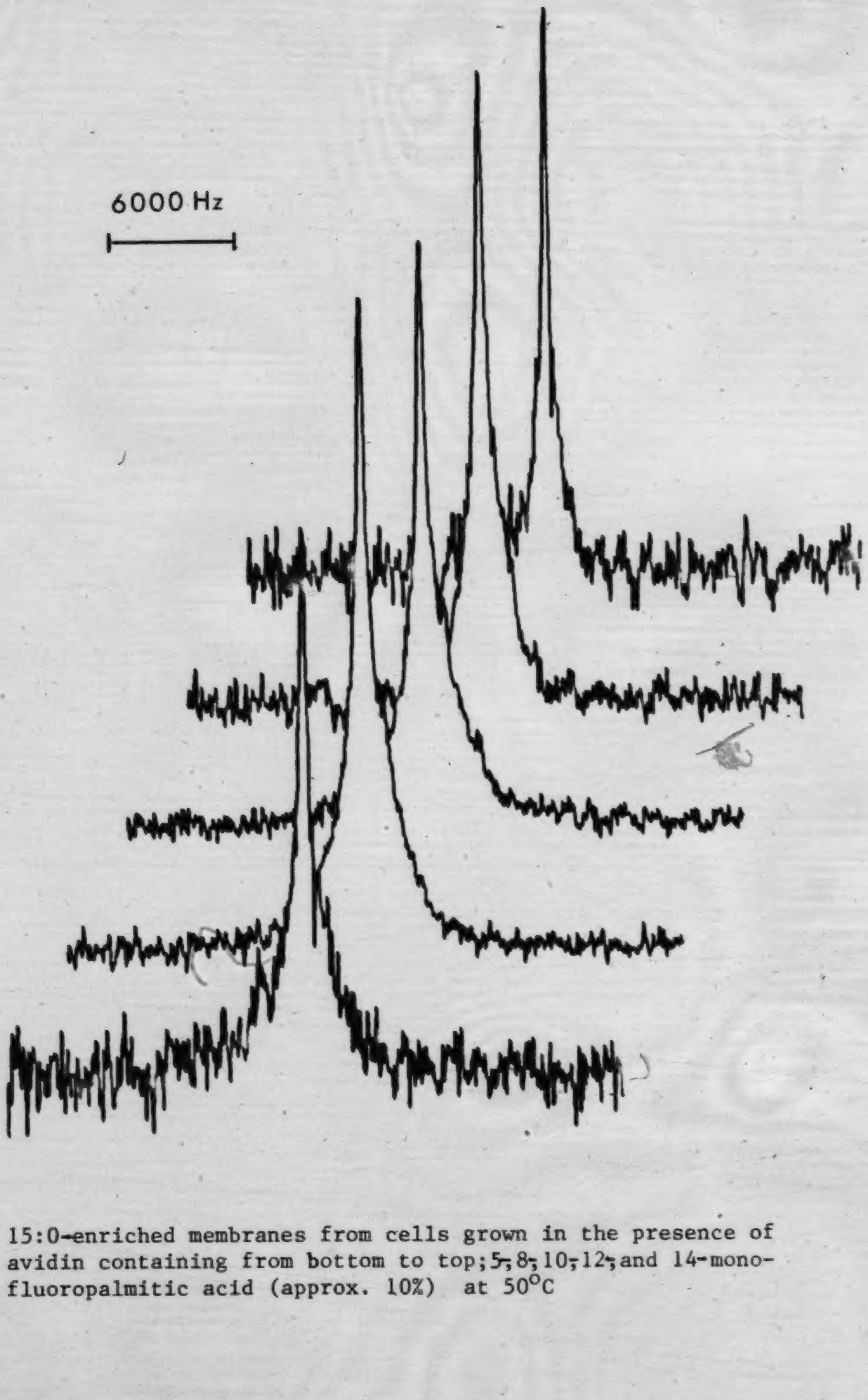
16:0-enriched membranes from cells grown in the absence of avidin containing from bottom to top; 5,8,10,12, and 14 monofluoropalmitic acid (approx.10%) at 45°C



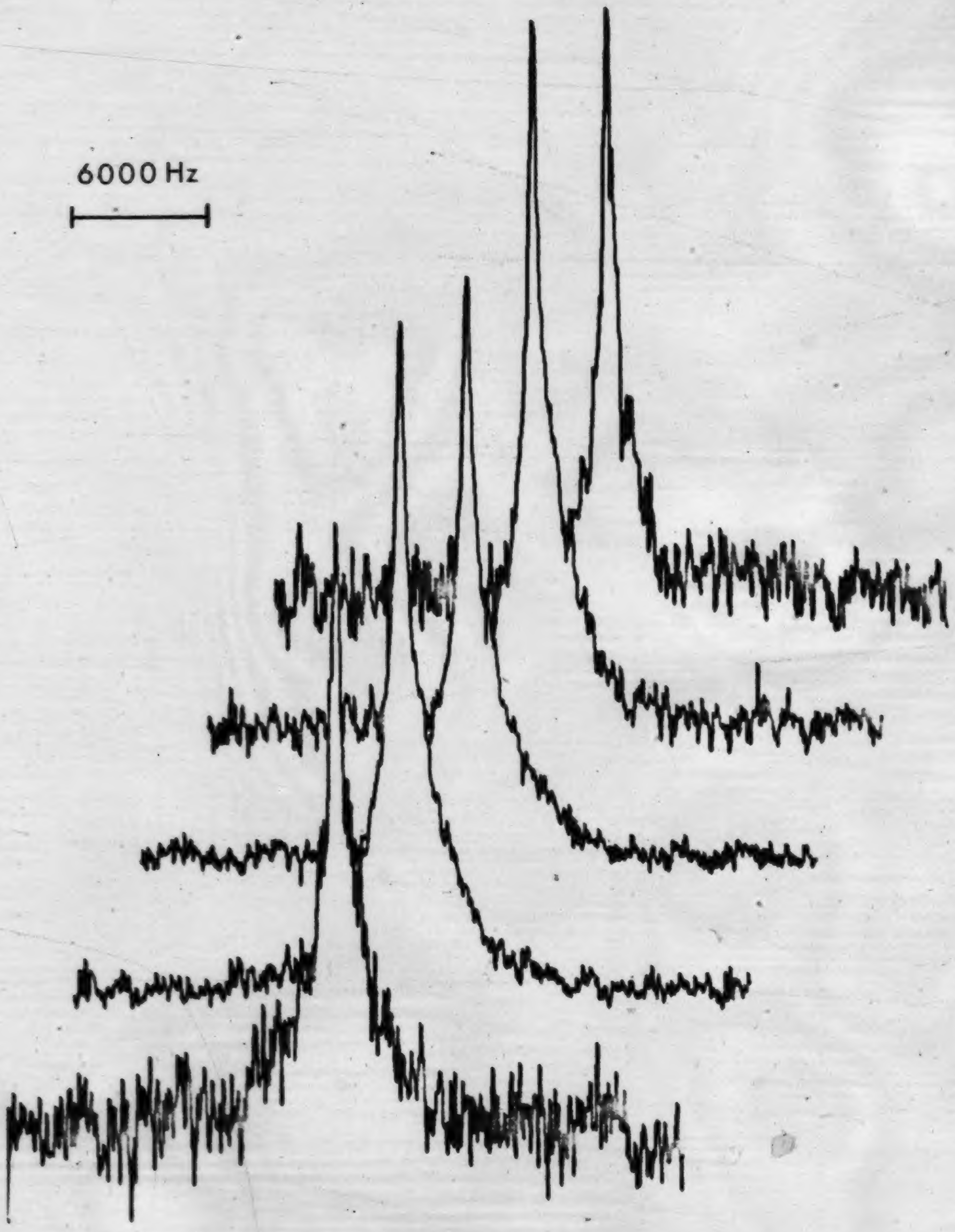
16:0-enriched membranes from cells grown in the absence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 37°C



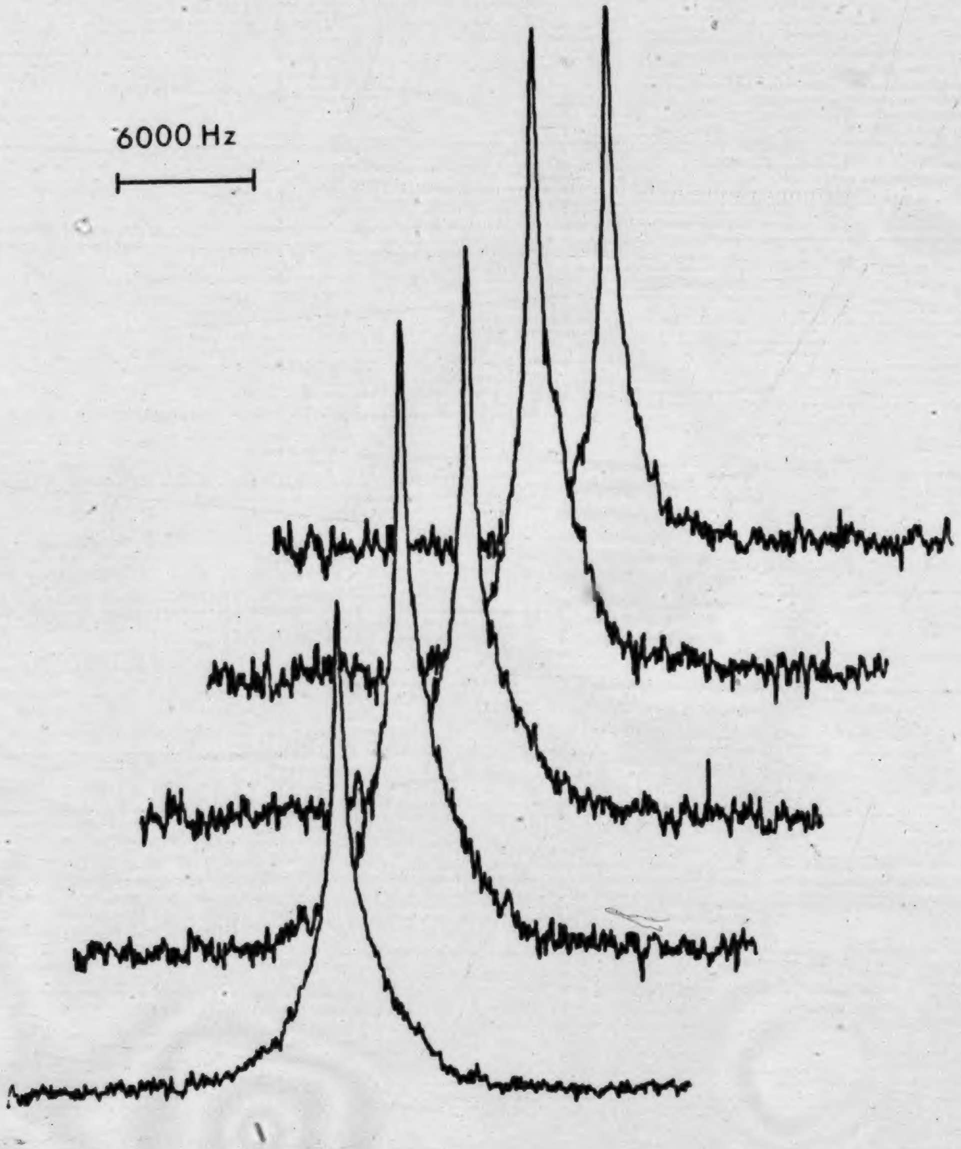
16:0-enriched membranes from cells grown in the absence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 20°C



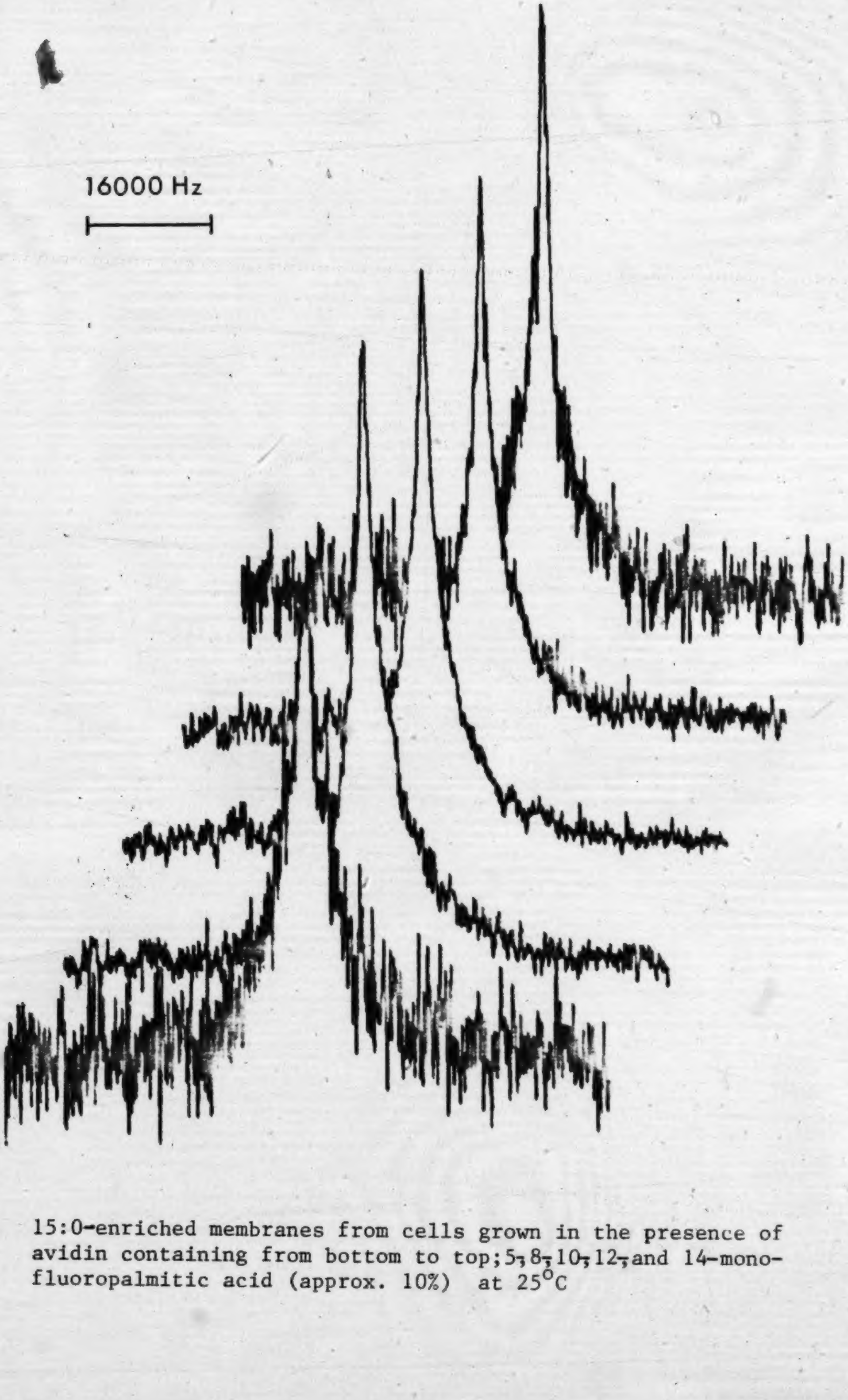
15:0-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 50°C



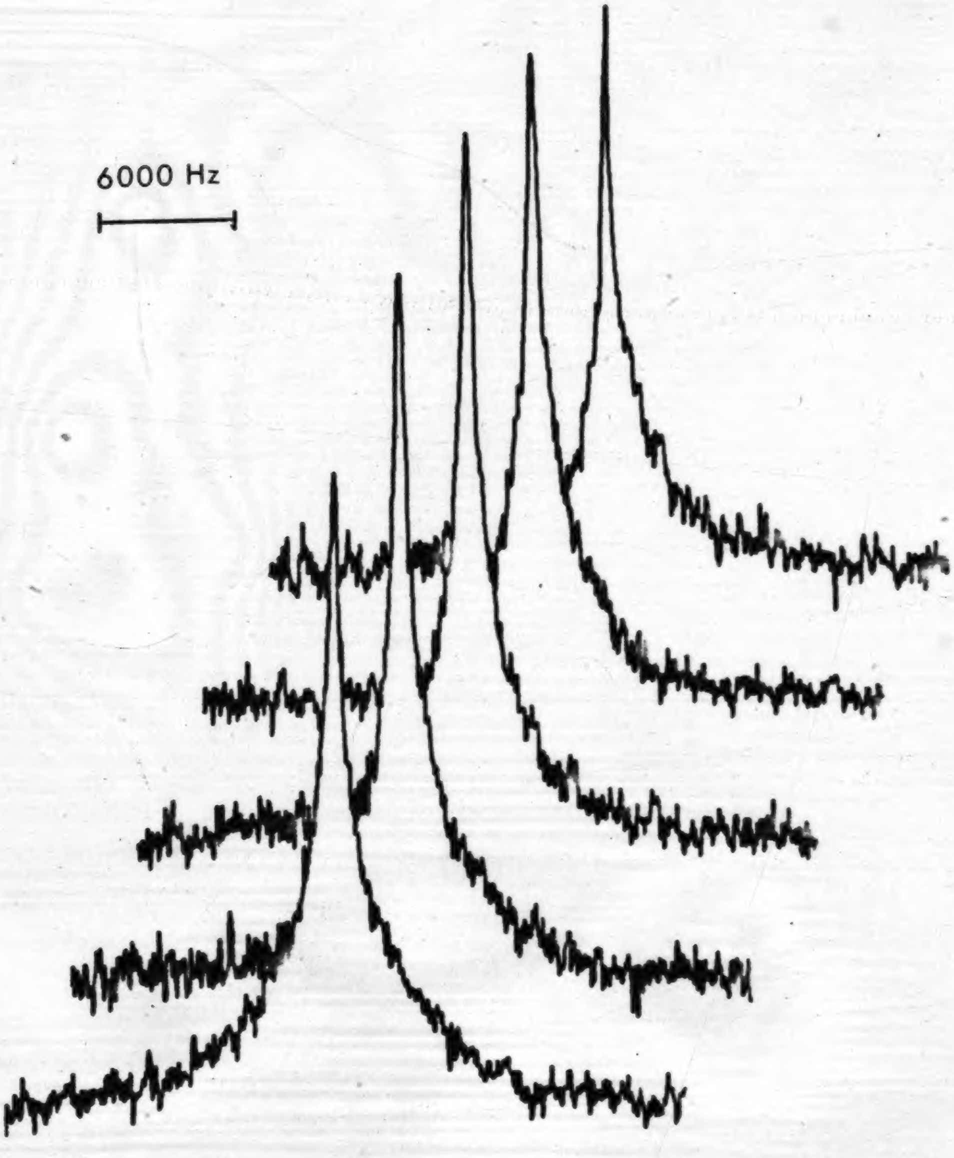
15:0-enriched membranes from cells grown in the presence of avidin containing from bottom to top;5,8,10,12,and 14-monofluoropalmitic acid (approx. 10%) at 37°C



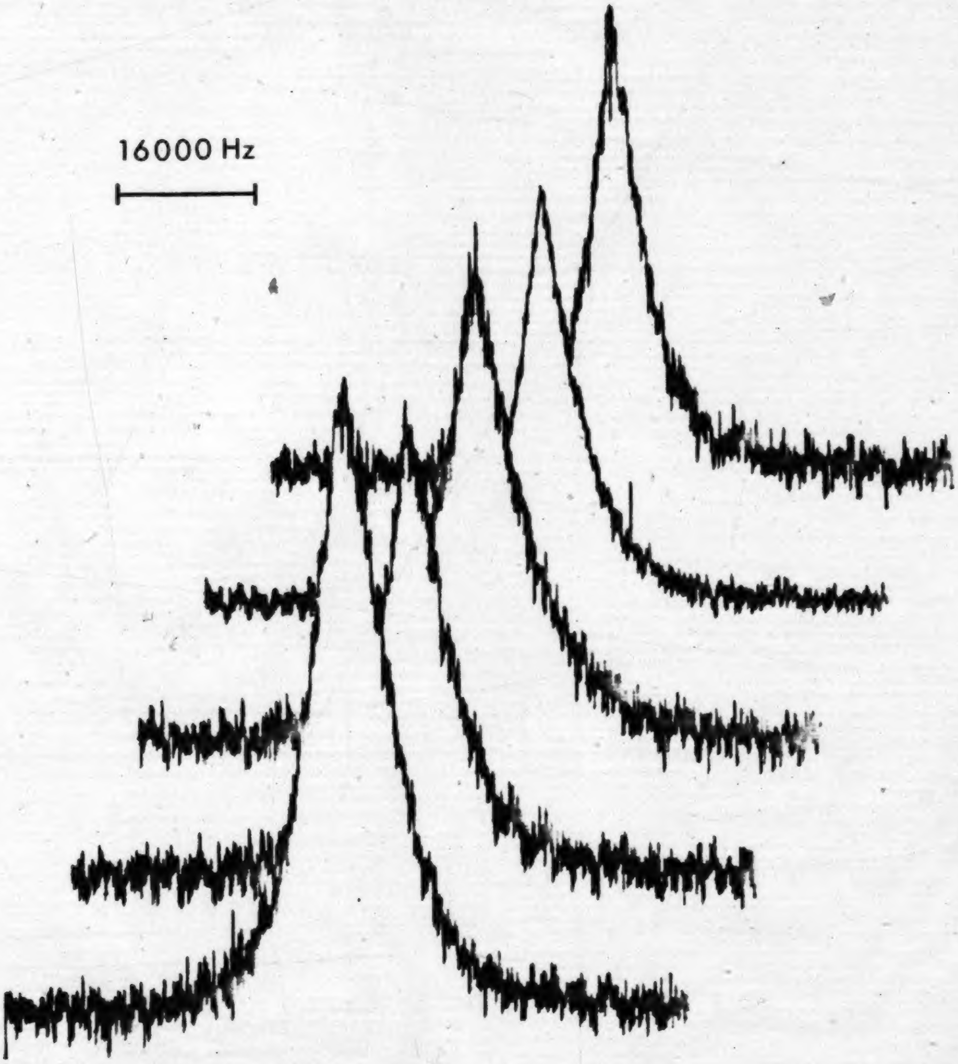
16:0i-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 37°C



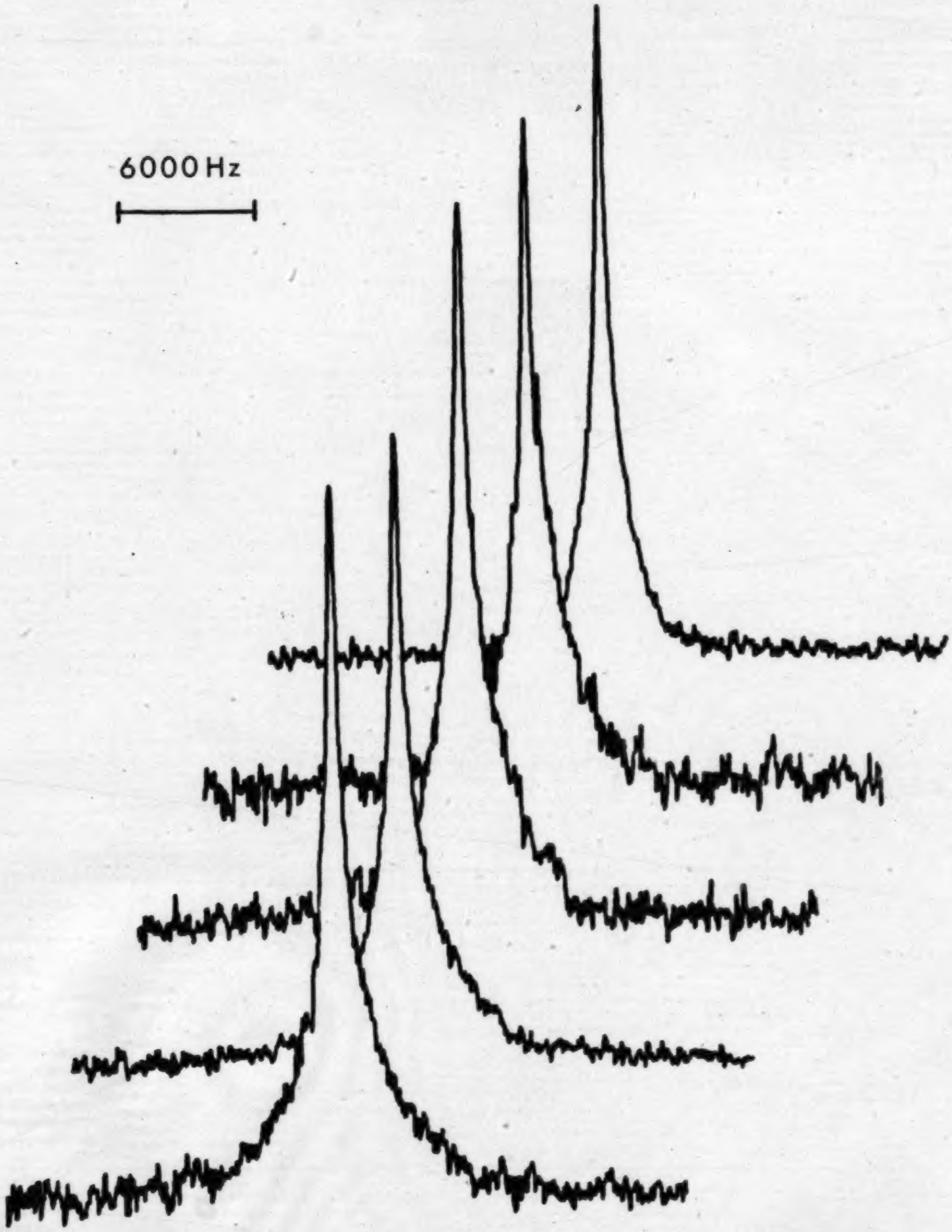
15:0-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 25°C



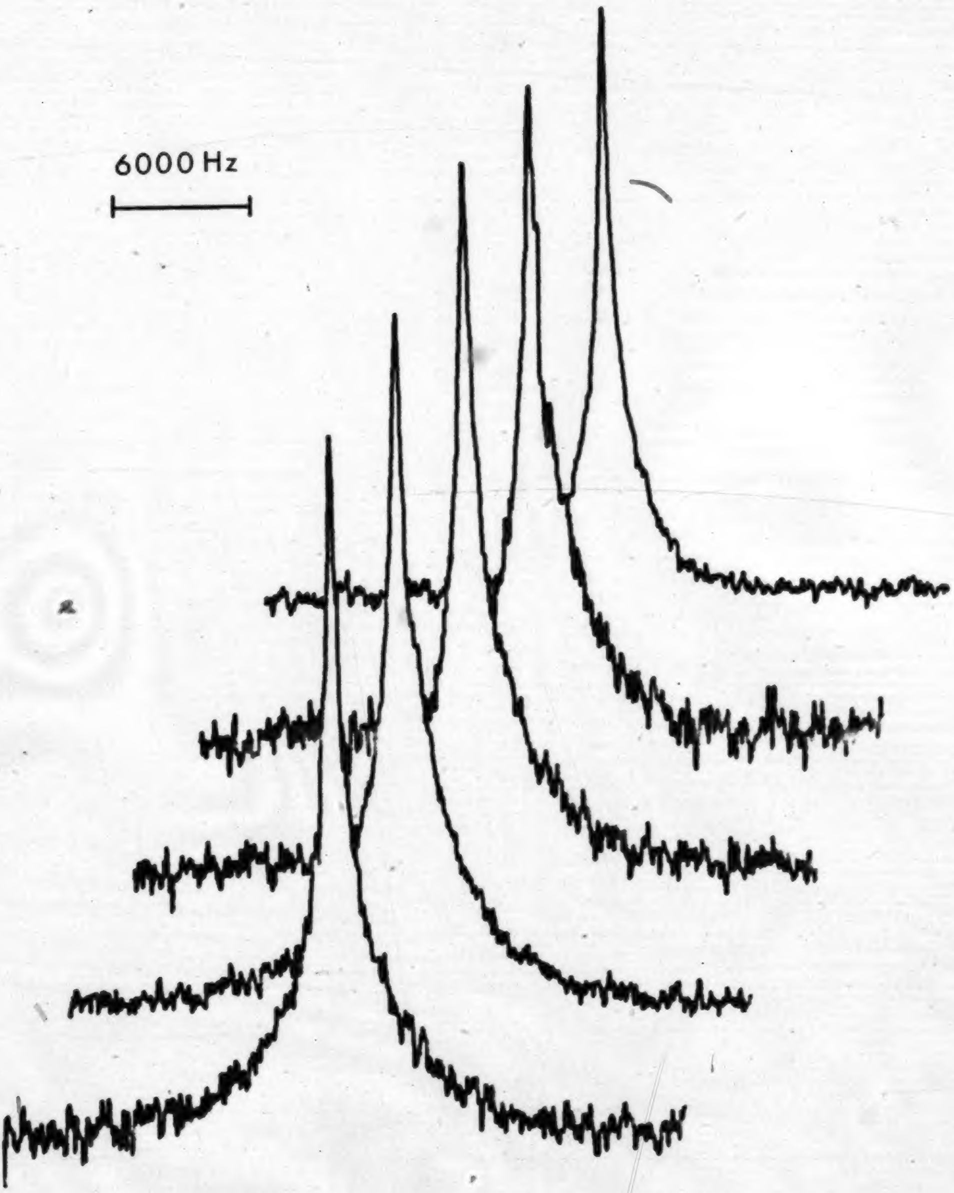
16:0i-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 25°C



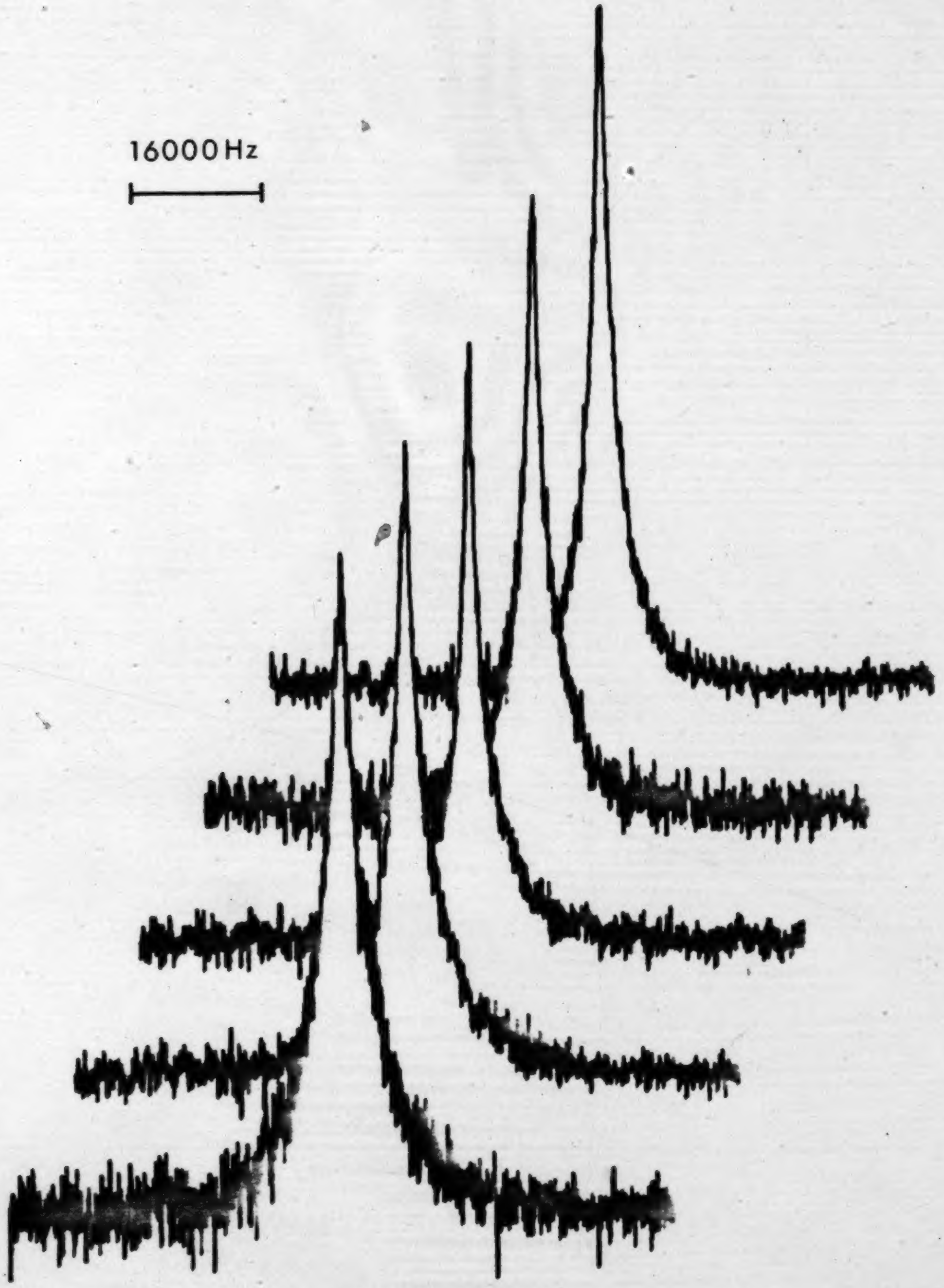
16:0i-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 10°C



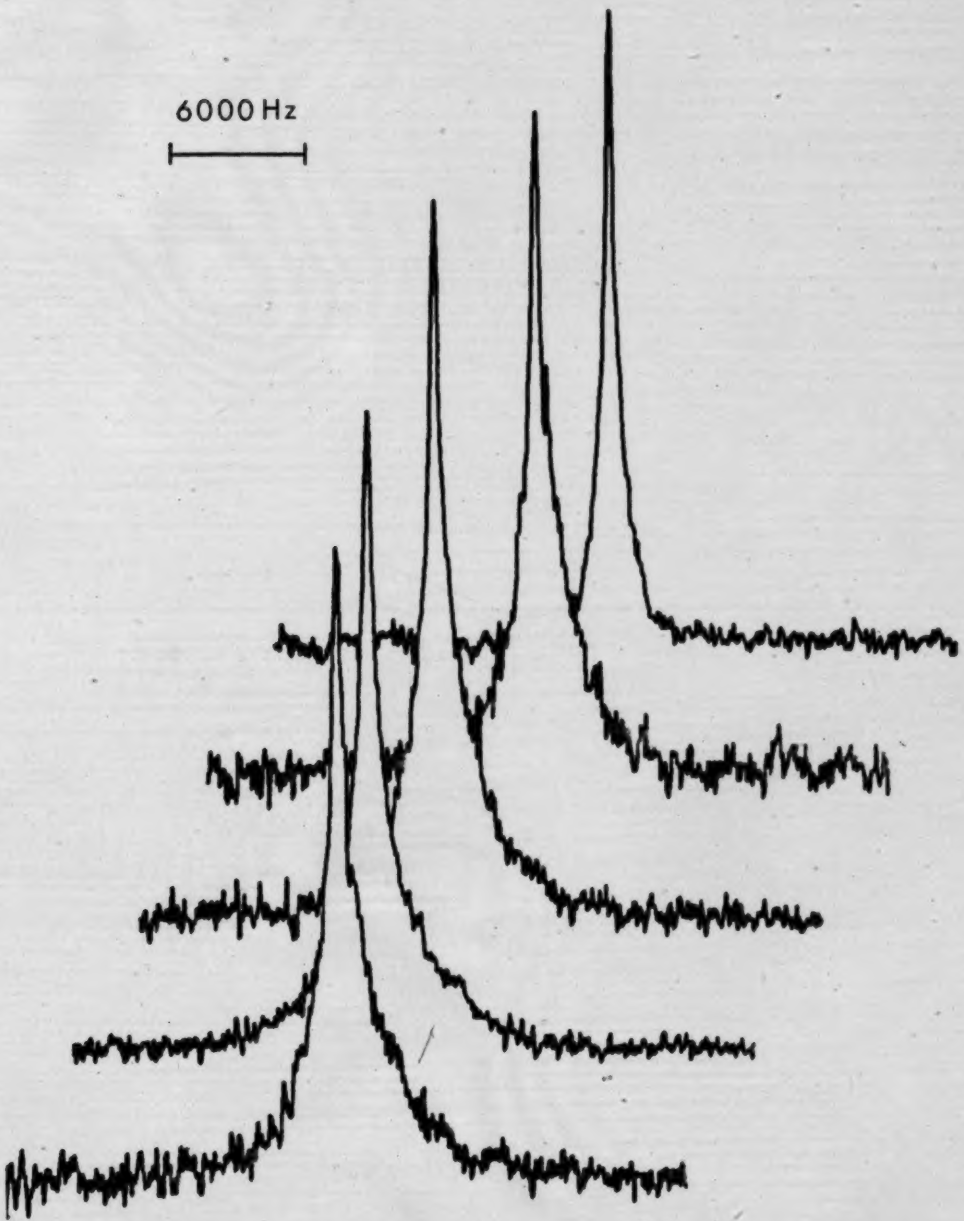
16:0ai-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at $37^{o}C$



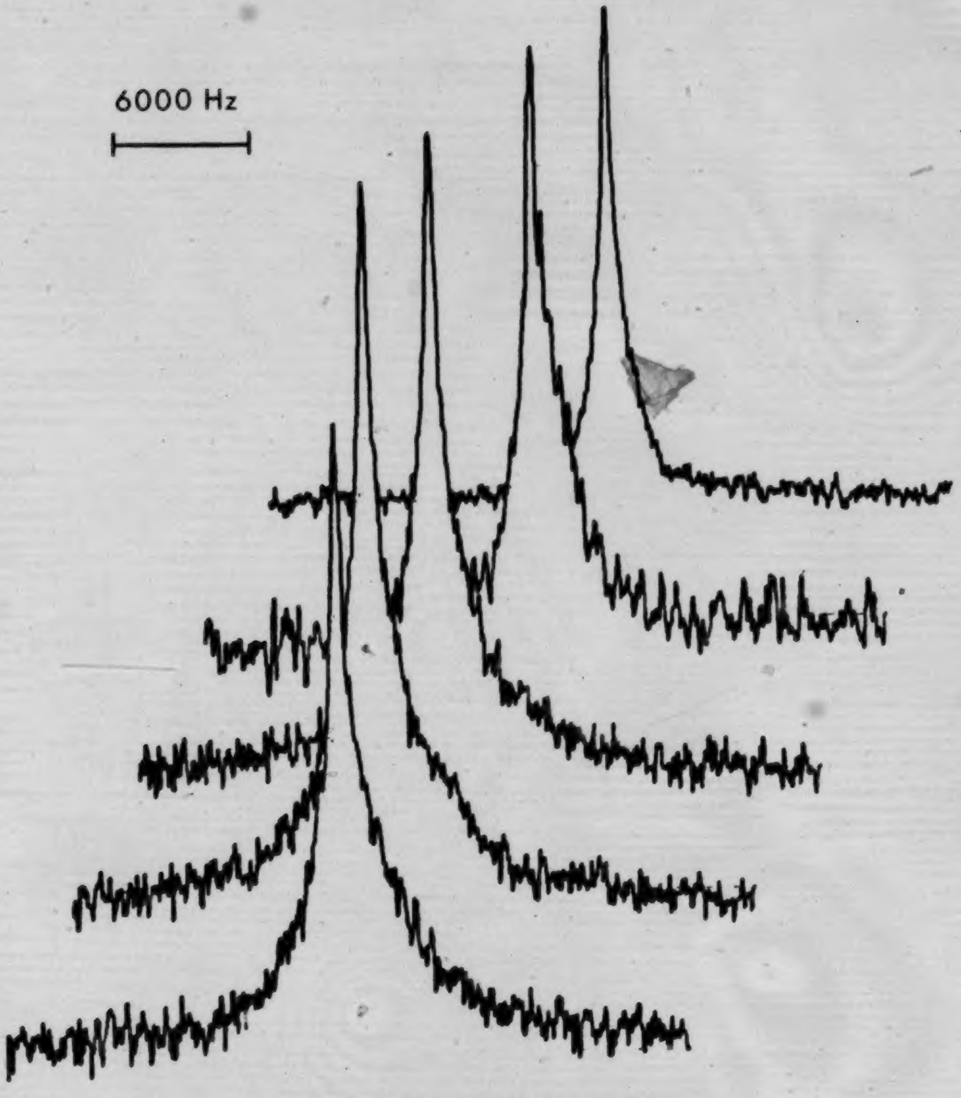
16:0ai-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 20°C



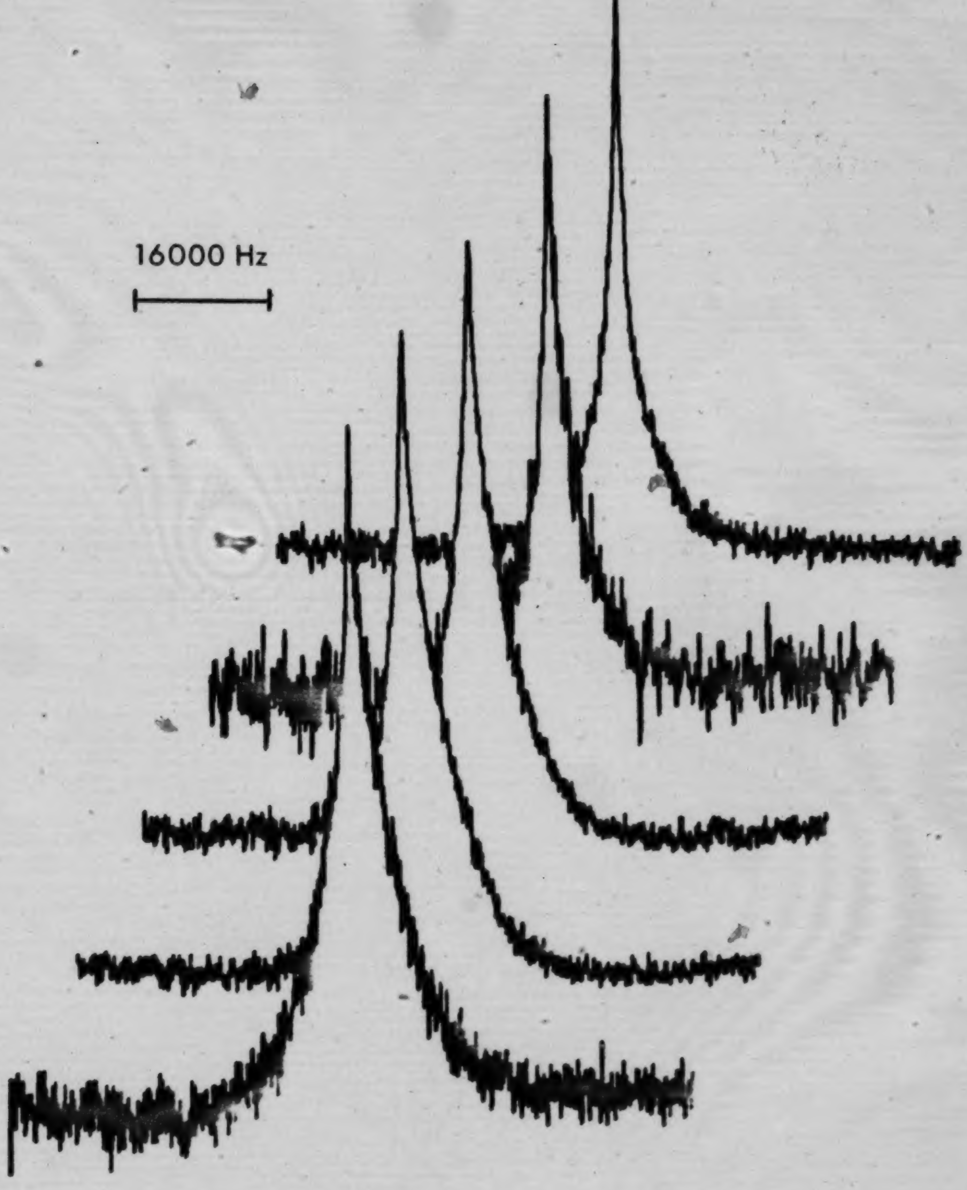
16:0ai-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 5°C



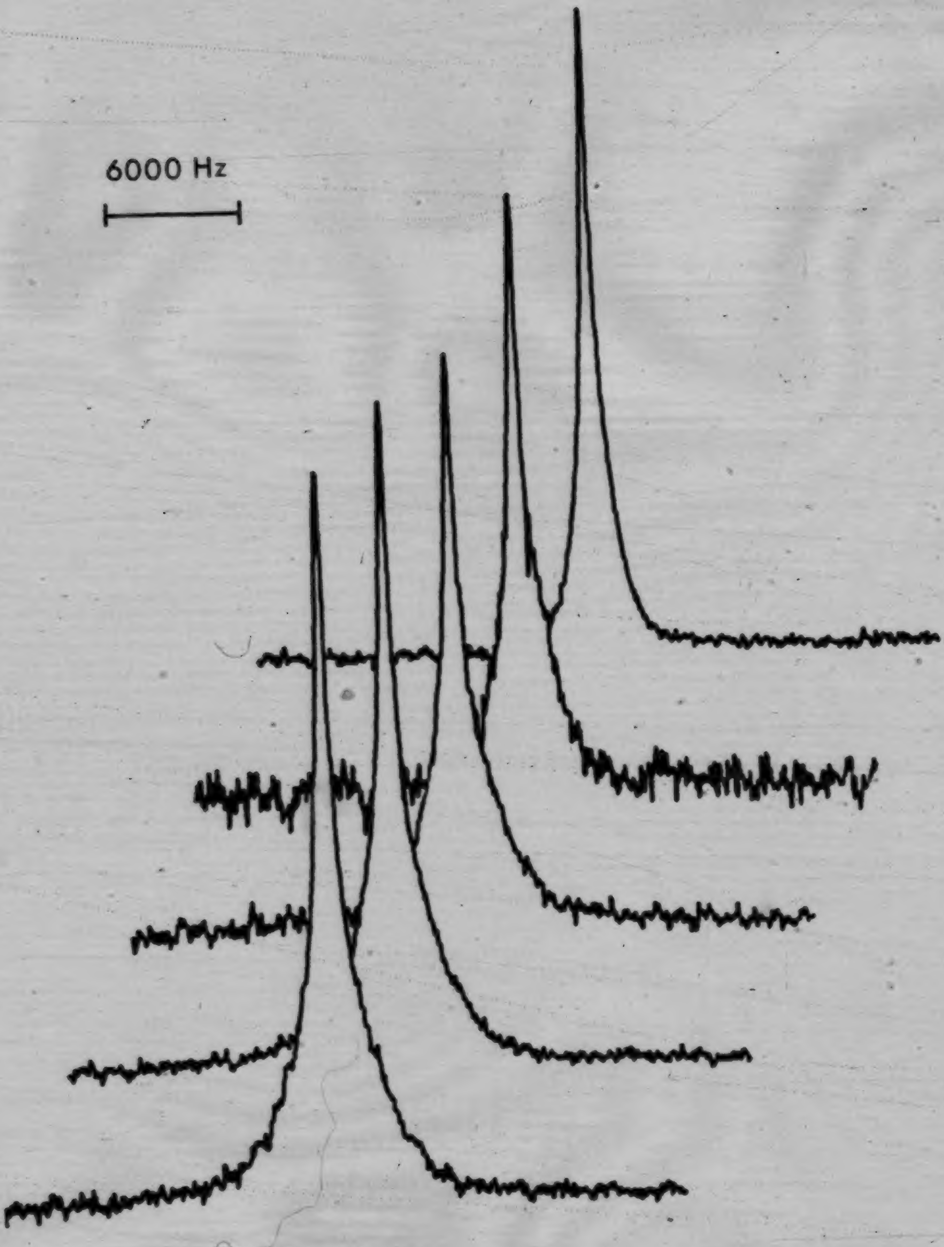
16:1tΔ^9-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 37^{o}C



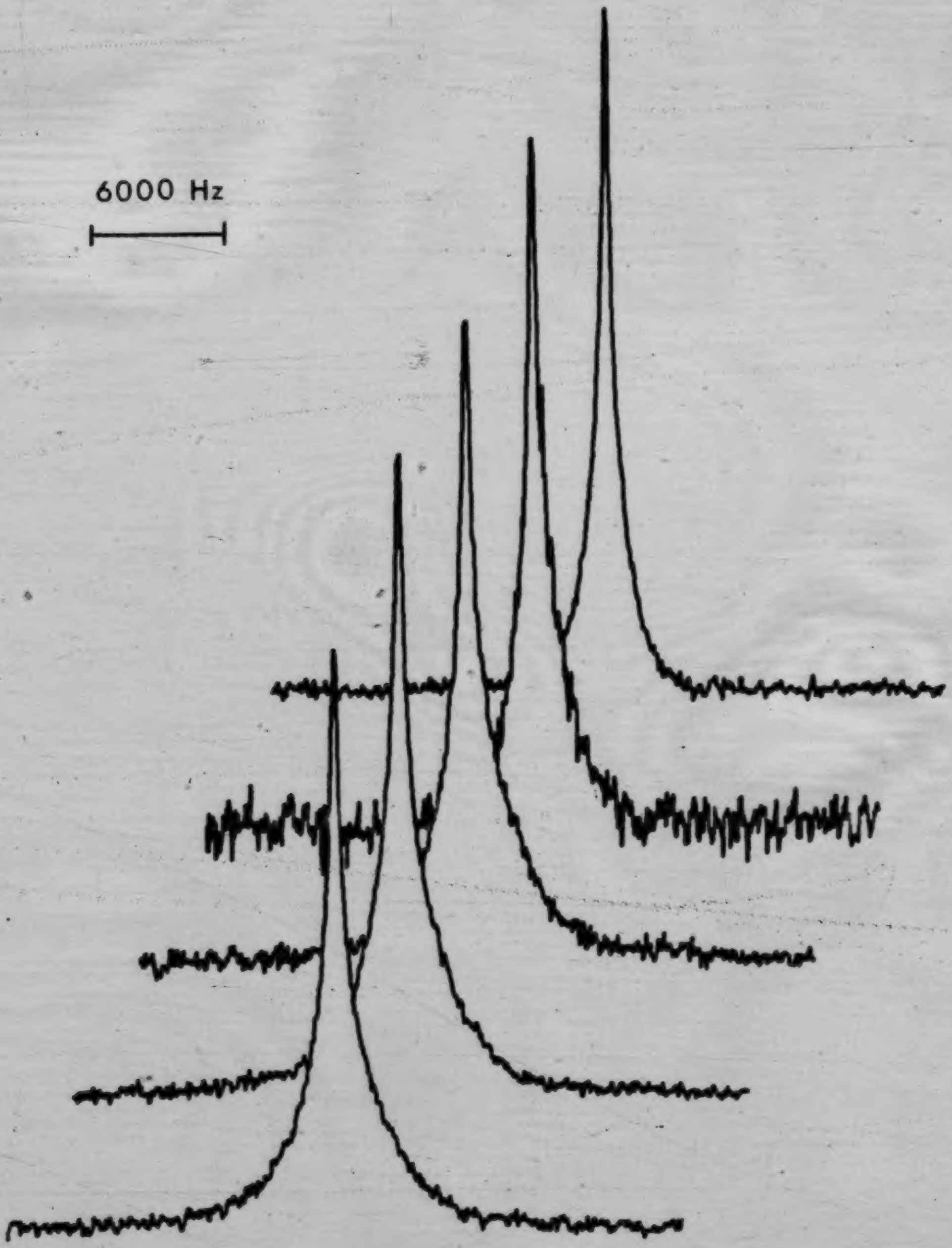
16:1tΔ^9-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5, 8, 10, 12, and 14-monofluoropalmitic acid (approx. 10%) at 20°C



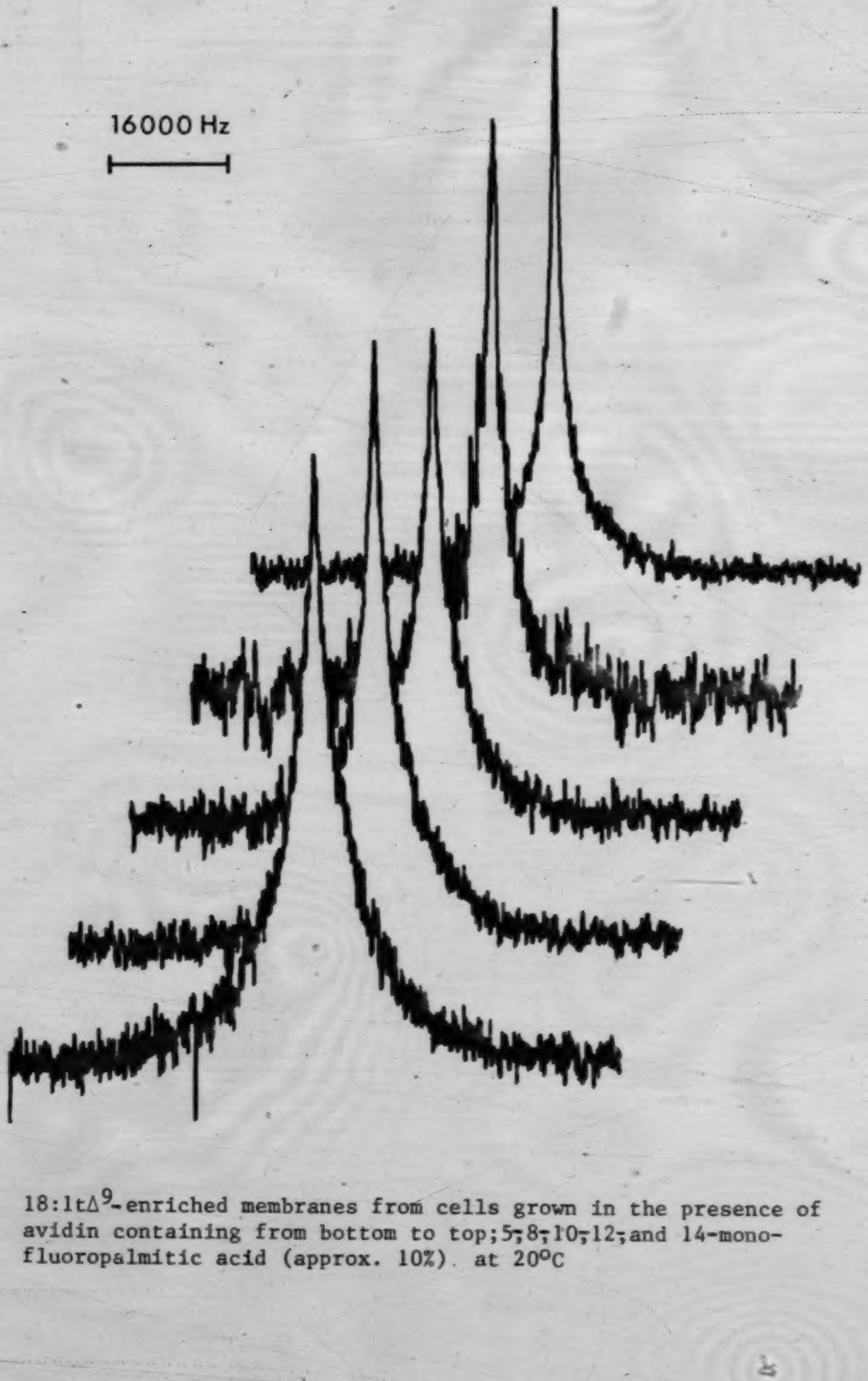
16:1tΔ^9-enriched membranes from cells grown in the presence of avidin containing from bottom to top;5-,8-,10-,12-and 14-monofluoropalmitic acid (approx. 10%) at 5°C



18:1tΔ^9-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 37°C



18:1tΔ^9-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%) at 34°C



18:1tΔ^9-enriched membranes from cells grown in the presence of avidin containing from bottom to top; 5-, 8-, 10-, 12-, and 14-monofluoropalmitic acid (approx. 10%). at 20°C